UNIVERSITY OF ALBERTA

EXPRESSION AND REGULATION BY SEX STEROIDS OF OXYTOCIN MESSENGER RNA IN RAT HYPOTHALAMUS AND HUMAN FETAL MEMBRANES.

BY

RAJNI CHIBBAR ©

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ABSTRACT

Oxytocin (OT) is an endocrine/paracrine hormone involved in many central and peripheral reproductive functions. It has been proposed to participate in regulating sexual maturation in the rat. In the first part of this thesis, I have examined the hypothesis that gonadal steroids influence the developmental increase of OT mRNA that has been demonstrated in both male and female rats. Northern blot analysis demonstrated that neural OT mRNA increased 5 to 10-fold from postnatal day 20 (P20) to P60 in both sexes, coincident with puberty. Pubertal upregulation of OT mRNA was largely but not completely inhibited by prepubescent gonadectomy, indicating a requirement for intact gonads as well as some other undefined factor(s). Pubertal treatment of gonadectomized animals with estradiol or testosterone restored neural OT mRNA similar to non-gonadectomized controls. Treatment of prepubertal animals with estradiol or testosterone from P10 to P20 had no effect on OT mRNA levels, suggesting that neural maturation or other factors are necessary requisites for steroid sensitivity. These data indicate that neural OT mRNA is induced by gonadal steroids during puberty, and suggest a mechanism for coordinating the development of reproductive function with other pubertal changes.

The endocrine role of OT in the initiation of parturition remains controversial. Although exogenous OT infusion will stimulate uterine contractions indistinguishable from normal spontaneous labour, maternal serum OT concentrations do not increase until labour has been well established. In the second part of this thesis I have examined the hypothesis that human intrauterine tissues (amnion, chorion and decidua) may synthesize OT mRNA, thus rationalizing a role for OT in the initiation of human parturition despite absence of an increase in peripheral circulating concentrations of OT. Using Northern blot analysis, *in situ* hybridization and ribonuclease protection assays, we demonstrated OT mRNA synthesis in all these tissues. Levels were highest in decidua which is situated immediately adjacent to the presumed target organ, myometrium. The transcript size in decidua is 60-80 nucleotides smaller than the transcripts in amnion and chorion. A significant increase (3-4 fold) in OT mRNA occurs in chorio-decidual tissues around the time of labour onset. Estradiol stimulates OT mRNA synthesis in *in vitro* incubations and this effect was partially inhibited by concurrent treatment with tamoxifen, suggesting that it is receptor-mediated, and with actinomycin D, it is due to increased transcription of OT gene. Progesterone has little, if any effect on basal synthesis of OT mRNA but largely prevents the estradiol-induced increase. We showed that both estradiol and progesterone receptor mRNAs are expressed in amnion, chorion and decidua. These results support the hypothesis of a paracrine system involving OT and sex steroids within intrauterine tissues that could rationalize a role for OT in the physiology of human labour. These data may lead to novel approaches towards prevention or treatment of preterm labour.

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LIST OF ABBREVIATIONS USED

Adreocorticotrophic Hormone (ACTH)
Activator Protein (AP)
Cerebospinal Fluid (CSF)
Cholecystokinin (CCK)
Corticotrophin Releasing Factor (CRF)
DNA Binding Domain (DBD)
Dalton (Da)
Diacylglycerol (DAG)
Diethylstilbesterol (DES)
Estradiol Receptor (ER)
Estrogen Responsive Element (ERE)
Follicle Stimulating Hormone (FSH)
Glucocorticoid Responsive Element (GRE)
Hormone Binding Domain (HBD)
Inositol triphosphate (IP_3)
Interferon Responsive Element (IRE)
Luteinizing Hormone (LH)
Messenger Ribonucleic Acid (mRNA)
Metabolic Clearance Rate (MCR)
Neurophysin (NP)
Norepinephrine (NE)
Nucleus of the Tractus Solitarius (NTS)
Oxytocin (OT)
Oxytocin Receptor (OTR)
Paraventricular Nucleus (PVN)

Phosphatidylinositol-4,5-biphosphate (PIP_2)

Phospholipase (PL)

Progesterone Receptor (PR)

Progesterone Responsive Element (PRE)

Prostaglandin (PG)

Prostaglandin H_2 Synthase (PGHS)

Retinoic Acid Receptor (RAR)

Signal Peptide (SP)

Standard Error of the Mean (SEM)

Supraoptic Nucleus (SON)

Thyroid Hormone Responsive Element (TRE)

Vasoactive Intestinal Peptide (VIP)

Vasopressin (VP)

Ventromedial Nucleus (VMN)

PART I

THE REGULATION OF OXYTOCIN GENE EXPRESSION DURING SEXUAL MATURATION OF THE RAT.

CHAPTER I

LITERATURE REVIEW AND BACKGROUND INFORMATION

1. INTRODUCTION

Oxytocin (OT) is a nonapeptide hormone synthesized principly in the magnocellular neurons of the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus. Axons from these neurons project to the posterior pituitary where the peptide is released into the peripheral circulation (Gainer et al, 1977; Silverman and Zimmerman 1983). Additionally, oxytocinergic *parvocelluar* neurons project to many extrahypothalamic brain areas, including regions of the spinal cord, brainstem, and forebrain (Sawchenko and Swanson 1982). In addition, several peripheral sites, including the gonads (Flint and Sheldrick, 1983a; Ivell and Richter, 1984a; Nicholson et al, 1984; Ivell et al, 1985; Guldennar and Pickering, 1985; Flint, 1986; Fehr et al, 1987; Ang et al, 1991; Foo et al, 1991), adrenal medulla (Ang and Jenkins, 1984; Nicholson et al, 1984; Nussey et al, 1987), placenta (Field et al, 1983; Makino et al, 1983; Lefebvre et al, 1992), The uterus (Ciarochi et al, 1985; Lundin et al, 1989), pancreas (Amico and Finns, 1988) and thymus (Geenen, 1986; Geenen, 1987)) synthesize OT which may regulate physiological functions in a paracrine and/or autocrine manner.

OT is involved in the physiology of many peripheral and central reproductive functions. In the periphery, OT is implicated in the regulation of ovulation, luteolysis, labour and milk ejection (Flint and Sheldrick, 1983; Freund Mercier and Richard, 1981; Gibbens and Chard, 1976; Mitchell et al, 1980; Ivell et al, 1985; Dawood et al, 1981; Viggiano et al, 1989; Fuchs et al, 1990) and centrally OT has a role in stimulating sexual and maternal behaviour (Pedersen et al, 1982; Arletti and Bertolini, 1985; Fahrbach et al, 1984,1985; van Leengood et al 1987, Arletti et al, 1987, 1990).

Sex-steroids are also of central importance in the reproductive processes. Higher circulating levels of estradiol have been correlated with plasma OT concentrations (Yamaguchi et al, 1979; Falconer et al, 1980). The higher levels of plasma OT in response to estradiol may be due to increase release from the posterior pituitary and/or may be associated with increased synthesis in the hypothalamic nuclei or peripheral organs (Robinson et al, 1976; Amico et al, 1981a, b; Mitchell et al, 1982; Amico et al, 1983; Caldwell et al, 1989; Shukovski et al, 1989; Tan et al, 1982; Ivell, 1985; Dawood et al, 1986). This effect also could be indirect secondary to an increase in an OT binding protein that may cause a decrease in clearance. However, a lag period of 10-24 hours between the administration of estradiol and peak levels of OT in the serum suggests that estradiol has stimulatory effects on OT biosynthesis (Robinson, 1974).

Hormonal regulation of OT gene expression *in vivo* is not well understood and controversial. A 1.5 to 2-fold increase in OT gene expression occurs in the supraoptic nucleus (SON) of female rats during estrous and correlates with higher levels of estradiol during proestrous (Van Tol et al, 1988). Conversely, ovariectomy of adult female rats decreases neural OT gene expression (van Tol et al, 1988; Miller et al, 1989; Burbach et al, 1990), suggesting that gonadal steroids or factors may regulate OT gene expression. However, treatment of ovarietomized adult female rats with estradiol had no effect on OT gene expression in the SON and PVN (vanTol et al, 1988; Burbach et al, 1990), suggesting that the mid cycle increase in circulating OT or higher OT gene expression during estrous may require other factors besides estradiol. Alternatively this could be due to technical problems, as a significant degree of expertise is required to remove the hypothalamic nuclei. It is not known how estradiol mediates its effects as the SON of female rats lack estradiol receptors and, in the PVN, only a subpopulation of oxytocinergic neurons which project centrally contain estradiol receptors (Pfaff and Keiner, 1973; Rhodes et al, 1982; Burbach et al, 1990). This may also suggest the possibility that effects of estradiol on OT gene expression may be indirect through other neurons that impinge upon oxytocinergic neurons.

Interestingly, direct effects of estradiol on OT gene expression *in vitro* is indicated by the presence of a functional estradiol responsive element (ERE) in the rat and human OT gene promoter (Richard and Zingg, 1990; Burbach et al, 1990). The effects of estradiol on OT gene expression are suggested to be tissue specific (Ivell et al, 1985) and were based on the indirect observations that ovarian OT gene expression varied cyclically whereas no such changes were observed in bovine hypothalamic OT mRNA. In later studies they showed that factors other than estradiol stimulate OT gene expression in bovine luteal cells. The bovine OT promoter does not contain a consensus ERE or respond to estradiol in *in vitro* transfection studies (Ivell et al, 1985; Furuya et al, 1991; Adan et al, 1991), suggesting that the OT gene is differently regulated in different species. Other evidence suggests that increased estradiol levels may not be necessary for increased OT gene expression. During lactation, OT mRNA remains at high levels while estrogen levels decrease after birth (Zingg et al, 1988). The few studies to date suggest that *in vitro*, estradiol stimulates OT gene expression but the *in vivo* effects of estradiol on OT gene expression are not clear.

It has been demonstrated that the OT peptide and mRNA increase in the hypothalamus during pubertal maturation in rats (Jackson and George, 1980; Miller et al, 1990). It is possible that increases in circulating sex steroid concentrations secondary to maturation of the gonads may be responsible for the increased biosynthesis of OT at this time. In the first part of this thesis I have examined the effects of sex steroids on OT gene expression during sexual maturation using classical endocrine/physiological protocols. The results of this study demonstrate that sex steroids play an important role in the upregulation of OT gene expression during sexual maturation.

1.1 HISTORICAL PERSPECTIVES

Historically, the uterotonic and milk ejection properties of the posterior pituitary extracts were first demonstrated by Dale (1906) and Ott and Scott (1910) respectively. For a long time only these two effects were known. Two major components- OT and vasopressin (VP) - were identified in the pituitary extract. Names were given on the basis of size and biological properties (Kamm et al, 1928).

Both OT and VP are synthesized mainly in the hypothalamic nuclei. Initial evidence of neurosecretion was obtained by using Gomori's stain, a stain specific for the cysteine-rich contents of secretory granules which identified axons of oxytocinergic and vasopressinergic neurons (Bargmann and Scharrer, 1951; Scharrer and Scharrer, 1954). These axons pass through the internal zone of the median eminence and end near blood vessels in the posterior pituitary gland where the peptides are released. These studies not only defined the hypothalamo-neurohypophyseal axis anatomically, but also introduced the now familiar concept of neurosecretion. Sensitive immunocytochemical studies have confirmed and extended these findings (Dierickx and van de Sande, 1979; Sofroniev, 1983).

2. BIOCHEMISTRY OF OT

2.1 BIOSYNTHESIS OF OT

Because of their close anatomical and chemical relationship, synthesis of OT has always been considered together with VP. Both peptides are synthesized together with a polypeptide of approximately 90 amino acids (DuVignault et al, 1953; North et al, 1980) termed *neurophysin*. In the 1940s it was considered that the neurophysin (NP), OT and VP formed parts of a larger protein - the Van Dyke protein- with a molecular weight of about 30 kDa, as all three existed together in pituitary acetone powders (Van Dyke et al, 1942). A *common precursor* hypothesis held that OT and associated NP is synthesized as a single prohormone (Sachs et al, 1969). Efforts to test this hypothesis proceeded at a relatively slow pace and subsequent successes were due to technical advances rather than conceptual ones. The identification of separate precursors for VP-NP and OT-NP was reported by Gainers and co-workers in 1977. ^{35}S cysteine was injected close to the SON and followed by a pulse-chase paradigm. Two separate 20 kDa proteins (OT-NP and VP-NP) were demonstrated by isoelectric focusing. Each protein contained a NP peptide. On limited proteolysis by trypsin, these 20 kDa precursors gave rise to NP, VP and OT. This was the first direct evidence in support of the common precursor hypothesis (Russell et al 1980). It was also shown that the VP precursor was glycosylated while the OT precursor was not.

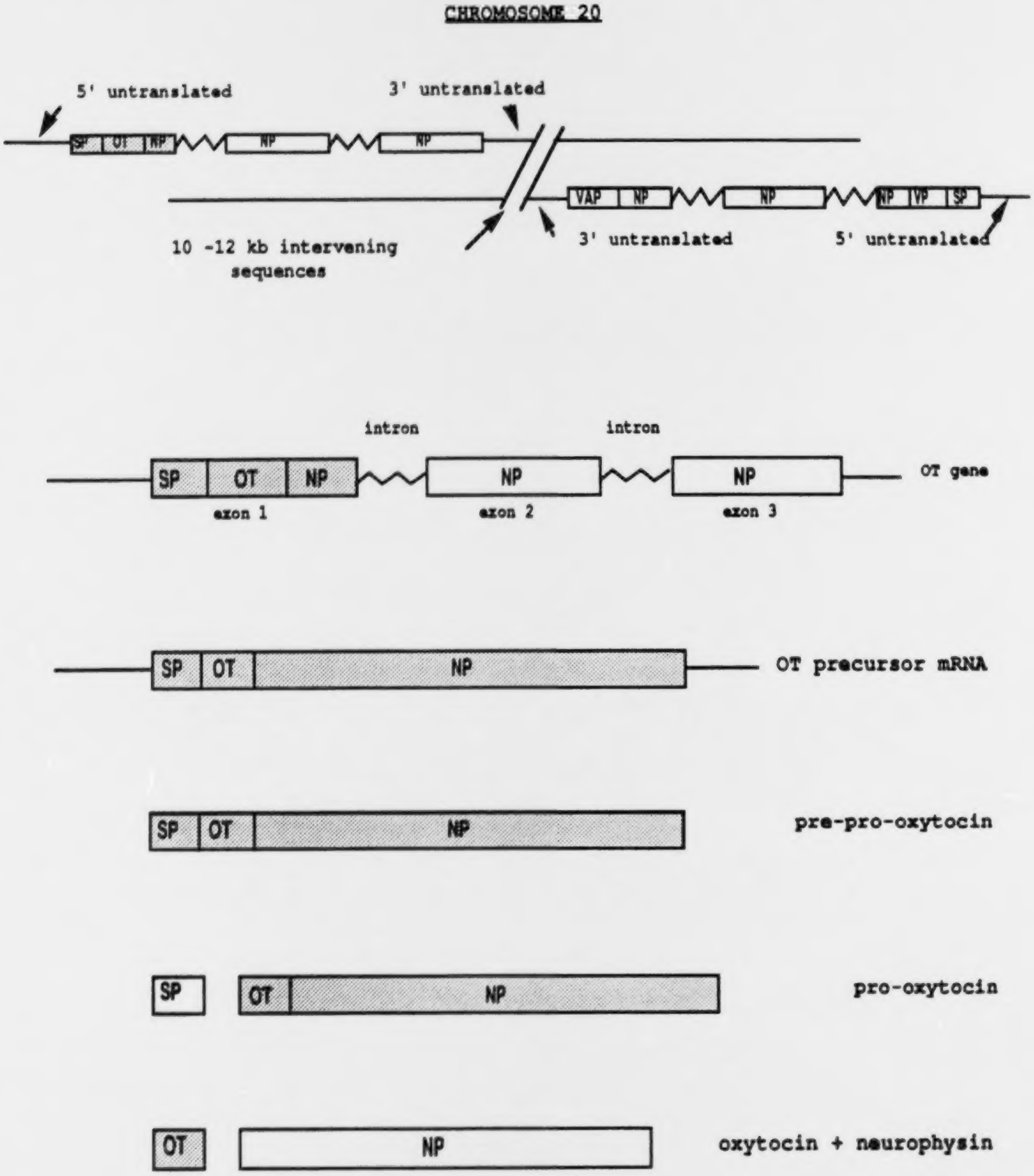
These *in vivo* experiments were confirmed by *in vitro* translation of bovine poly A^+ RNA and cDNA cloning and showed that OT is made via a precursor which includes a signal peptide, OT and neurophysin I (pre-pro-OT) (Schmale et al, 1979; Schmale and Richter 1980; Land et al, 1983). The pre-pro-OT is 16.5 kDa and pro-OT is 15.5 kD in size. The molecular weight of OT is 1 kDa

2.2 GENE STRUCTURE OF OT

Despite the high degree of sequence homology OT and VP are the products of independent genes. The genes for OT and VP are similar in their intron-exon structure and are closely linked, separated by only twelve kilobase pairs. They exist in opposite transcriptional orientations on chromosome 20 (Ivell and Richter, 1984b, Sausville et al, 1985; Mohr et al, 1988a). The OT gene consists of three exons and two introns. Exon-1 encodes for the 19-amino acid signal peptide, the nonapeptide OT and the first 9 amino acids of NP-1. OT and NP-1 are separated from each other by three basic amino acids Gly-Lys-Arg that is recognized by specific proteolytic enzymes. Exon-2 encodes for the central 67 amino acids of NP-1 and exon-3 encodes for the remaining 17 amino acids of NP-1 and the terminal arginine.

High sequence similarity exists between OT and VP in exons A (80%) encoding OT and VP nonapeptides and in exons B (96%) encoding the central core of neurophysin. This level of homology is consistent with a common evolutionary origin for both genes. Exon

Figure I-1. Schematic representation of the oxytocin (OT) and vasopressin (VP) genes along with their associated neurophysins (NP) and signal peptides (SP)on chromosome 20.



C is the least homologous between the two genes. It encodes for the remaining NP for both OT and VP and, in the case of VP, an additional 39 amino acid glycopeptide (VP associated glycopeptide) (Richter, 1983; 1987,1989; Rehbein et al, 1986).

The 5′ untranslated region of the OT gene contains a RNA polymerase II binding site 68 nucleotides upstream from the translation start site (Ivell and Richter, 1984). The promoter region of the OT gene contains several potential estrogen (ERE) and glucocorticoid (GRE) responsive elements, suggesting that this gene is under steroid regulation (Mohr et al, 1988). An imperfect (GGTG*ACCTTGACC), but fully functional, ERE starting at position -164 is present in the human gene (Richard and Zingg, 1990; Burbach et al, 1990; Adan et al 1991). Two additional sequence elements that correspond to the right half of the ERE (TGACC), are present at positions -103 and -83. These sites are required for the binding of thyroid and retinoic acid receptors. Both the ERE and the "half-ERE" have been conserved at similar positions in the promoter region of the rat OT gene (Ruppert et al, 1984; Hara et al, 1990). In the rat OT promoter two imperfect GREs, which may be negatively or positively regulated by glucocorticoids, are also present at positions -2392 and -2256. At -170 the OT gene shares homology with a negative regulatory sequence motif of the human polyoma virus BK enhancer which binds a HeLa cell specific potential repressor. This sequence element overlaps partially with the ERE of the OT gene, suggesting that positive and negative tissue specific transcriptional factors may be involved in the expression of this gene. Recently, Lipkin et al (1992) demonstrated that the ERE of the rat gene also binds the retinoic acid receptor with high affinity and functions as a negative retinoic acid response element.

Sequence analysis of the OT promoter also revealed two activator protein-2 (AP-2) sites identical to the AP-2 sequence motif of the plasminogen activator gene and growth hormone genes indicating possible regulation by protein kinase-A and protein kinase-C signal transduction pathways. A putative interferon-responsive element (Mohr et al, 1988a) and multiple octanucleotide enhancer sequences (the significance of which is yet unknown) are also present. Factors regulating transcription of the OT gene seem to be diverse and tissue specific, as hypothalamic and ovarian regulation of the OT gene is shown to be independent of each other.

The 3′ untranslated region of the rat gene contains three polyadenylation signals (AATAAA). The significance of these is not known. In rat hypothalamic cDNA, addition of the poly A tail occurs at base 837 downstream of all polyadelynation sites. Bovine luteal OT mRNA is smaller than OT mRNA of hypothalamic origin. This tissue-specific size heterogeneity of OT mRNA is due to a difference in the poly A tail length (Ivell and Richter, 1984b; Carter and Murphy, 1989) and may represent a mechanism for regulation of gene expression at the post-transcriptional level.

2.3 OT STRUCTURE and PROCESSING

Neurosecretory granules represent the major subcelluar organelles responsible for converting neuropeptide precursors into their biologically active components. Pro-OT, which is the functionally inactive hormonal precursor, undergoes a series of processing steps in the secretory granules during its axonal transport from the hypothalamus to the posterior pituitary. The nonapeptide sequence occupies the amino terminal domain of the proform and is separated from the NP-1 by a triplet of amino acids (Gly-10, Lys-11, Arg-12). The Gly-10 is involved in intramolecular carboxy-terminal amidation of Gly-9 (Bradbury et al, 1982). The Lys-11 and Arg-12 act as signals for further proteolytic processing. Post-translational processing of the OT precursor requires an endoprotease, with an apparent molecular weight of 58 kDa. This enzyme cleaves at the Arg-12/Ala-13 peptide bond, releasing a carboxy-terminal basic residue that separates OT from its carrier protein (NP-1) to form oxytocinyl-GKR (gly-lys-arg). Oxytocinyl-GK and then oxytocinyl-G are formed by a carboxypeptidase B, which is specific for OT (Eipper et al, 1985, 1987; Eipper and Mains, 1988). The amidating enzyme converts the resulting oxtocinyl-G into functionally active OT (Perkins et al, 1990). Neurophysin modulates the rates of maturation of oxytocin precursor and protects it from enzymatic action of carboxypeptidase B in *in vitro* (Ando et al, 1988). The processing of pro-OT in the secretory granules of the corpus luteum is analogous to that in the brain

Figure I-2. Processing of oxytocin (OT)-neurophysin I (NP) to form the biologically active OT-amide (OT-NH2)

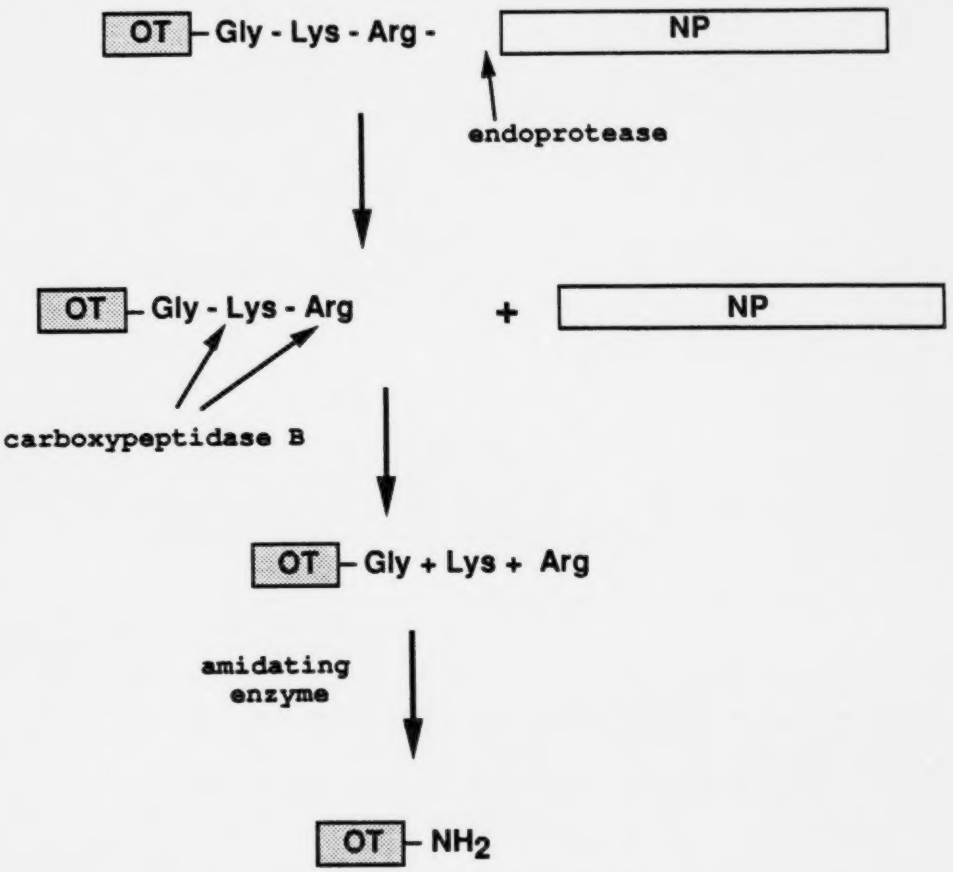
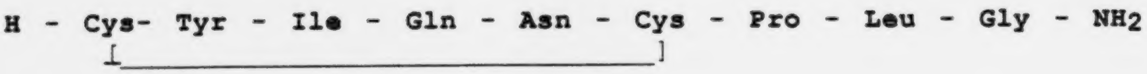


Figure I-3. Chemical structure of oxytocin.

OXYTOCIN



$\mathrm{H - Cys - Tyr - Ile - Gln - Asn - Cys - Pro - Leu - Gly - NH_2}$

(Plevrakis et al 1988; Sheldrick and Flint, 1989). However, heterogeneity in the intraneuronal post-transcriptional processing of the OT precursor in estradiol treated primates has been demonstrated (Amico and Hemple 1990). Estradiol treatment stimulates release of the OT-precursor into the circulation of humans and monkeys. Estradiol therefore may exert an effect upon the post-translational cleavage of the OT prohormone, the significance of which is not yet known.

OT consists of 9 amino acid residues, two of which are cystines forming a disulfide bridge between residues 1 and 6 (fig 3). The biological effects of OT are dependent upon the integrity of the resulting ring structure. Reduction and alkylation of the disulfide link causes complete loss of biological activities. The tyrosine at position 2 is also essential for full biological potency. Analogues with substitutions at this site are much less active.

2.4 BIOTRANSFORMATION AND METABOLISM

OT modulates a variety of brain functions (DeWeid, 1983; Pedersen et al, 1982). The central activity of OT is not limited to its complete nonapeptide structure. Presence of aminopeptidase activity in the brain at the putative site of OT action converts OT into potent smaller neuroactive peptides. The *Aminopeptidase* activity predominates in synaptic membrane (Burbach et al, 1980, 1981) and cleavages from the amino terminus shows a sequential pattern of peptide fragments resulting in (Cyt6)-OT 2-9, (Cyt6)-OT 3-9, (Cyt6)-OT 4-9 and (Cyt6) OT 5-9. The major OT fragment generated by brain synaptic membranes is a hexapeptide pGlu-Asn-Cys(cys)-Pro-Leu-Gly-NH_2 ((cyt 6)-OT 4-9)). This peptide and its des-glycinamide derivative was found to be 100 fold more potent than OT in attenuating memory functions but was completely devoid of uterotonic activitiy (Burbach et al 1983). VP is also degraded by the aminopeptidase activity in a similar manner (Burbach et al, 1987). However, although the mechanism of conversion is similar, marked differences exist in the rates of conversion. OT is more resistant to proteolytic cleavage than VP. The half-life of intact OT is about 5 fold longer than that of VP under identical experimental conditions. This difference in susceptibility to aminopeptidase cleavage is primarily determined by the amino acid residue at position 3 (Burbach et al 1982).

In the periphery, OT is metabolized in the liver and kidney (Koida et al, 1971; Vorherr and Kleeman, 1979). The catabolic pathways are similar to those in the brain. The metabolism of OT during pregnancy is discussed in chapter 2, section 3.

2.5 EVOLUTIONARY PERSPECTIVES

The VP/OT hormone superfamily is widely distributed throughout the animal kingdom, including groups as diverse as mammals to invertebrates (Acher and Chauvet 1988). All peptides of this family contain a nine amino acid peptide backbone with several highly conserved residues, including the two cystines at positions 1 and 6 that forms the disulfide bridge (Morley et al 1990).

In all non-mammalian vertebrate species, *vasotocin* has been chemically identified in place of VP, while in non-mammalian tetrapods and bony fish OT is replaced, respectively, by *mesotocin* and *isotocin*. In these species vasotocin/isotocin regulates water and salt metabolism (Sawyer and Pang 1979). Recently the cloning of cDNAs encoding the mRNA for vasotocin, mesotocin/ isotocin and the two vasotocin genes from toad, white sucker and teleost fish have been reported (Nojiri et al, 1987; Heierhorst, 1989; Morley, 1990). These studies suggest that the structural organization of the mesotocin and vasotocin precursors are highly homologous to those of the OT and VP precursors, respectively. The neurophysins of vasotocin and isotocin are extended by 30 amino acids at their C-termini, and these extensions show striking similarities with the glycopeptide of the VP precursor, except that they lack glycosylation sites. Based on the sequence homologies among the precursors of the VP/OT family it has been hypothesized (Heierhorst et al 1989) that OT and VP evolved from an ancestral molecule, about 400 million years ago, by gene duplication of the vasotocin gene. This gene encodes the nonapeptide and a larger neurophysin protein. During the course of evolution the C-terminal part of neurophysin became a separate glycopeptide with distinct biological functions (VP precursor) or was simply discarded because of lack of functions (OT precursor).

3. OT IN THE CENTRAL NERVOUS SYSTEM

3.1 ANATOMY OF THE OT NEUROSECRETORY SYSTEM

Oxytocin is synthesized in the oxytocinergic neurons of the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus. These neurons project to the posterior pituitary where the peptide is released into the peripheral circulation (Silverman and Zimmerman 1983). In many animal species the SON consists of a relatively homogeneous group of magnocellular neurons that project entirely to the neural lobe of the pituitary (Zimmerman 1981). The SON contains many more magnocellular neurons than does the PVN and is thus, quantitatively at least, more important in determining the output of OT and VP to the peripheral circulation. Some of the neurons from the SON terminate in the external zone of the median eminence and secrete into the long portal vessels. The concentration of OT is 10-15 times higher in portal blood than in the peripheral circulation (Gibbs 1984). OT and VP are synthesized in separate neurons (Dieriekx and van de Sande, 1979; Buijs et al, 1978; Sofrnoniev, 1983).

By contrast the PVN contains a large number of different cell groups only some of which are magnocelluar. It contains some parvocellular cells that are smaller, and less well defined compared to magnocellular neurons. Many of its OT and VP cells project to sites other than the neural lobe. VP and OT neurons from the rostral part of PVN project to the posterior pituitary.

The caudal part of PVN projects to the autonomic centers of the medulla and spinal cord, especially to the nucleus of the tractus solitarius and the dorsal vagal nucleus (Buijs et al, 1978; Sawchenko and Swanson, 1982).

A large number of so-called accessory magnocellular OT neurons are scattered in various groups throughout the hypothalamus. Considerable species variation exists in their precise location, but several groups are present in most mammals, including humans. The localization of various groups of oxytocinergic neurons in the rat brain by *in situ* hybridization (Burbach et al, 1987; Miller et al, 1989) shows the presence of extrahypothalamic oxytocinergic neurons in various regions. These regions include the rostral forebrain and the vicinity of the bed nucleus of stria terminalis, an area important in brain memory function. The OT gene is expressed at approximately similar levels at various sites in the brain. The functions of these dispersed groups of oxytocinergic neurons is not known. Different groups appear to have different projections and are sensitive to different stimuli (Cohen et al 1988).

Recently, the presence of OT and VP mRNA in the posterior and neurointermediate lobe of the pituitary has been demonstrated (Mohr et al, 1990; Jirikowski et al, 1990). The origin of OT mRNA in the posterior pituitary is not known. It may be either synthesized in the posterior pituitary or transported from the oxytocinergic neurons. However, Mohr et al (1990) could not detect any OT mRNA in the posterior pituitary of the rat after disconnecting the hypothalamo-pituitary tract, suggesting the latter possibility. It has been suggested that this may be a mechanism for regulation of OT mRNA in hypothalamus.

3.2 ONTOGENY OF OT

The cells that form the SON and PVN arise in the germinal epithelium, which lines the diamond shaped region of the third ventricle. From there, cells migrate laterally to form the PVN and ventrolaterally to form the SON (Altman and Bayer 1978). The NP-containing magnocellular neurosecretory cells in the rat cease cell division between embryonic day 13 and 15 (E13-E15) (Iffet, 1972; Anderson 1978). The precursors for both OT-NP-1 and VP-NP-2 are detected at E16 (Almazan et al 1988; Alstein et al 1988). Further increases in both the number of cells and intensity of staining occur between E16-postnatal day 9 (P9) (Whitnall et al 1985). The adult-like distribution of oxtocinergic and vasopressinergic cells in the PVN and SON is apparent by E17. In the PVN, the oxytocinergic cells are peripherally distributed and vasopressinergic cells form the central core. NP-OT-containing axons are detectable in the median eminence at E18-E19 (E17 for VP). Alstein and Gainer (1988) showed that only the OT precursor and C-terminal extended forms of OT are present before birth and the amidated form of OT is assayable only after birth. A marked lag exists between the appeareance of the amidated form of VP (E16-E17) and that of OT (E21-E22). This suggests that there may be a significant developmental delay in the biosynthesis and processing of OT precursor compared to that of the VP precursor. The biological significance of this delay is not known. In the human fetal pituitary, OT is present at 14-17 weeks at the levels of about 10 ng/ gland and increases during gestation to about 300-400 ng in 1-5 day old infants (Khan-Dawood and Dawood, 1982).

The pituitary levels of OT are low after birth in rat pups and then increase between day 8 and 21 (Sinding et al, 1980). The hypothalamic OT precursor peptide and mRNA increase around the time of pubertal maturation (Jackson and George, 1980; Miller et al, 1990).

3.3 COEXISTENCE and COLOCALIZATION WITH OTHER PEPTIDES

The concept that a neurone may release more than one active substance is expanding in all areas of nervous system. One of the initial applications of immunohistochemical technique to the study of hypothalamic neuruhypophyseal system was to investigate whether VP and OT were produced in the same or in separate neurons. Diericks and van de Sande (1979) showed in various mammals that neurons contain either VP and its associated neurophysin or OT and its associated neurophysin. These results were confirmed by Mohr et al (1988b) using *in situ* hybridization with double labelled cRNA probes. This may imply that, in vasopressinergic hypothalamic cells, the OT gene is maximally supressed while in oxytocinergic cells the VP gene appears to be dormant. Recently, evidence for the coexpression of OT and vasopression mRNA in magnocellular neurosecretory cells during lactation has been presented (Jirikowski et al, 1989).

Other peptides have been described in the magnocelluar neurons and may coexist with OT in the same neurons. OT coexists with vasopressin, cholecystokinin, atrial natriuretic factor, substance-P and met-enkephalin, galanin (Martin and Voigt, 1981; Martin et al, 1983; Jirikowski et al, 1989; Kaltwasser and Crawley, 1987; Vanderhaeghen et al, 1981; Landry et al 1990) in the same granules. The functional significance of the coexisting peptides in neurohypophyseal secretion is not known. However, enkephalin inhibits OT release at the nerve terminals by acting on opioid receptors or via interactions with the neurohypophyseal dopaminergic pathway (Bondy et al, 1989).

OT neurons which contain corticotropin releasing hormone (CRF) peptides are present in both magno- and parvocellular nuclei (Pretel, 1990). The functions of CRF are associated with stress behaviour and OT release has also been associated with stress conditions e.g restraint stress (Bruhn et al, 1986). In addition, OT and CRF stimulate the release of placental pro-opiomelanocortin peptides (Margioris et al 1988), and OT appears to potentiate the ACTH releasing activity of CRF, while CRF seems to increase the release of OT under stressful conditions (Gibbs et al, 1984). Thus, the importance of coexistence of OT and CRF might be their combined participation in modulating stress induced responses. A role for their coexistence has been implicated in the stimulation of neurohypophyseal hormone release through an interaction with the intermediate lobe of the pituitary (Bondy et al, 1989).

3.4 OT RECEPTORS IN THE BRAIN

The action of OT is mediated by specific high-affinity binding sites for OT in brain (Brenton et al, 1984; DeKloet et al, 1985; van Leeuwan et al, 1985; Jhonson et al, 1988). The functional OT binding sites in fetal rat brain first appear at E14 in a region that later differentiates into the dorsal motor nucleus of the vagus (Tribollet et al, 1988). The OT binding sites later appear in different regions of brain between E20 to P3. From P5 to P16 the distribution of OT binding sites remain unchanged, while the density of binding increases in all labeled structures and reaches maximum around P10 to P13.

A change from the infant pattern to the adult pattern occurs around the time of pubertal maturation. In the hypothalamic ventromedial nucleus (VMN, the region of brain involved in sexual behaviour) OT binding is almost undetectable until P40, the approximate time of pubertal maturation, and attains its adult pattern around P60 (Tribollet et al, 1988). This region also contains a high density of estrogen receptors in adults (Pfaff and Keiner, 1973; Stumpf et al, 1975). This expression therefore is probably dependent on gonadal steroids, which are synthesised in great amounts from this period of development onwards. Estradiol administration to ovariectomized female rats increases OT receptor numbers in the VMN, suggesting the neural circuits in such regions may become sensitized to central OT via estradiol-dependent modulation of OT receptors (De Kloet et al, 1986; Johnson et al, 1989a,b). Together these data suggest that the increase in OT and estradiol during pubertal maturation play an important role in the modulation of sexual behaviour through their respective receptors.

3.5 REGULATION OF OT SYNTHESIS AND SECRETION

3.5.1 Regulation at the neurohypophysis

Despite the fact that action potentials generated in the cell body of magnocellular neurons are primary determinants of the ultimate release of OT, studies have indicated that electrical events originating in cell bodies do not represent the final stage of neural integration of OT secretion. OT release from nerve terminals is subjected to a number of modulating influences at the neurohypophysis level by neurotransmitters, neuropeptides or OT itself. These factors could be present in the fibers running in to the posterior pituitary, could coexist in the same nerve terminals as the neurohypophyseal hormones or could be synthesised in the intermediate lobe. The manner in which neurotransmitters affect OT release is not clear, yet one possibility is that modulating axons may synapse at preterminal sites on neurosecretory neurons and modulate the release of OT.

Dopamine is the most abundant amine in the neurohypophysis and stimulation of the dopaminergic system inhibis OT release (Vizi and Volkebas, 1980). The dopaminergic fibers originate in the arcuate nucleus of the hypothalamus (Baumgarten et al, 1972) and terminate in close contact with neurosecretory axons and pituicyte processes. Dopamine binding sites are present in the neurohypophysis (Cronin and Weiner, 1979), suggesting the effects of dopamine on OT release could be direct.

Gamma-aminobutyric acid (GABA) also inhibits electrical and potassium-stimulated OT release from the posterior pituitary (Dyball and Shaw, 1978; Holtzbauer et al, 1978) and this effect appears to be receptor mediated, both in male and female rats (Anderson and Mitchell, 1986; Saridaki et al, 1988).

Similarly to dopamine and GABA, an inhibitory role of opioid peptides has been shown by various investigators (Haldar et al, 1978; Bicknell and Leng, 1982; Bicknell et al, 1983; Zhao et al, 1988; Cheng et al, 1990; Rossier et al, 1979; Clarke et al, 1979).

The role of the adrenergic neurotransmitter system in regulation of OT release at the neurohypophysis is not known. In the neural lobe α-1 and α-2 adrenergic receptors have been identified and characterized. The α-1 adrenergic receptor appears to be present on pituicytes and/or blood vessels rather than on neurosecretory fibers. It has been suggested that a substantial part of posterior pituitary NA has its origin in sympathetic fibers originating in the superior cervical ganglion, suggesting a functional role for the peripheral nervous system in the control of neurohypophyseal blood flow and/or functions.

The well known pharmacological stimulus for the secretion of OT and its associated neurophysin in the human is the administration of estrogen (Amico et al, 1981), and this will be discussed in detail in section 3.5.5.

3.5.2 Regulation in the hypothalamus

Numerous putative neurotransmitters and neuropeptides have been localized in the magnocelluar neurons and are believed to play a modulatory role in OT synthesis and secretion. Considerably more work is required to elucidate the mechanism of action and physiological significance of these factors which are briefly described below.

Besides actions at the tuberohypophyseal pathway, dopamine also have inhibitory effects at the hypothalamic level in the regulation of OT secretion (Clarke et al, 1979; Moos and Richard, 1982; Barker et al, 1971).

The role of the central noradrenergic system in regulating OT secretion is implicated in the mediation of suckling, vaginal distention and electrical stimulation of the vagus (Tribollet et al, 1978; Moos and Richards 1975; Guzek et al, 1981). The adrenergic system exerts dual control over the release of OT. A number of electrophysiological studies have indicated that the principal action of norepinephrine (NE) on the SON and PVN neurons is excitatory (via α-1 receptors). At the same time there is some evidence for an inhibitory action of NE which may be mediated by other receptor types (α-2 and β) (Barker et al, 1971; Lefcourt and Akers, 1984; Inenaga et al, 1986; Carter and Lightman, 1987; Song et al, 1988).

The role of acetylcholine in the release of OT is not clear. There are studies suggesting that systemic or topical application of acetylcholine causes release of OT from the hypothalamus as well as from bovine granulosa cells (Abraham and Pickford, 1954; Dreifuss and Kelly, 1972; Bridges et al, 1976; Luck 1990).

In summary, the regulation of OT secretion in the hypothalamo-neurohypophyseal axis is complex and the net OT secretion is a result of the interaction of various stimulatory and inhibitory neurotransmitters, neuropeptptides and steroids at the oxytocinergic neurons or in other neurons which impinge upon the oxytocinergic neuronal system.

3.5.3 Neuronal plasticity

Adult brain is capable of structural plasticity in response to lesions as well as to prolonged physiological stimuli. During parturition, lactation or prolonged dehydration, oxytocinergic neurons undergo reversible structural changes (Theodosis and Poulain, 1987). The specific changes are a marked decrease in the glial coverage of oxytocinergic neurons and the juxtaposition of almost every OT secreting neuron, at one point or another along its surface, to adjacent cell bodies and /or dendrites (Theodosis et al, 1986; Chapman et al, 1986). These changes result in coordination of electrical activity and synchronization of high frequency discharge in oxytocinergic neurons. Coincident to neuroglial changes the dendrites, which usually contact single post synaptic elements, are also observed to synapse onto two or three different postsynaptic elements simultaneously (shared synapse). This synchronizes neuronal firing by allowing several postsynaptic elements to receive the same information simultaneously.

Structural changes are also observed in the posterior pituitary (Twiddle and Hatton, 1987). Under basal conditions, neurosecretory axons are surrounded by pituicytes, a type of astrocyte. Pituicyte processes also occupy much of the perivascular space. Upon prolonged stimulation, pituicytes recede from between adjacent neurosecretory terminals and from the perivascular space and a greater terminal surface area is left in contact with the blood vessels.

The cellular mechanisms underlying these plastic changes are not clear and both neuronal and glial elements appear to be involved. OT itself has also been suggested to play a role in this structural plasticity. Prolonged intracerebroventricular infusion of OT or its agonists, in normal rats induce the same changes as those seen under physiological stimulation (Theodosis et al, 1986). Increased peripheral release of OT may be associated with the central release (CSF or release into magnocellular neurons itself) of OT, which in turn would affect the anatomy of the neurons that release it. Once stimulation ceases, glial processes and pituicytes reappear (Theodosis and Poulin, 1984).

3.4.5 Peripheral stimuli

Several peripheral stimuli like suckling, feeding and ejaculation also influence OT release, indicating that OT levels can be elevated in response to activation of a variety of sensory nerves originating in different regions of body (Forsling et al, 1979; Fuchs et al, 1984; Ogawa et al, 1980). Cranial nerves IX and X relay in the nucleus of the tractus solitarius (NTS). Fibers from the NTS relay either in α-1 noradrenergic nuclei, which in turn project to magnocellular neurons, or to parvocellular neurons of the PVN. Afferent stimuli of vagal nerves produce an increase in OT levels in rats (Moos and Richards, 1979). Vagal reflexes triggered during feeding may lead to a release of OT which in turn may influence gastrointestinal motility and endocrine secretion e.g. insulin and glucagon (Verbalis et al, 1986; Amico et al, 1988). An increase in OT release in response to sensory stimuli may facilitate milk let down during lactation. A positive feedback system for OT exists during parturition, where cervical stretch by the descending fetus provides a feed forward system for further OT release, to stimulate additional uterine contraction. The exact mechanism by which these stimuli affect the oxytocinergic system is not well understood.

Steroids, thyroid hormones and retinoic acid

Estrogens are known to stimulate the release of OT and its associated neurophysin from the neurohypophysis in primates and humans (Legros and and Franchimont, 1975; Robinson, 1974; Robinson et al, 1976, 1977). The increase in plasma OT and NP occurs within several hours (10-12) of estrogen administration and could be due to increased release of preformed OT and NP and/or new synthesis.

The effects of estradiol on OT biosynthesis (discussed in section 1) are supported by the demonstration of direct regulation of the OT promoter by estradiol in various cell lines by gene transfection studies. Unfortunately none of these cell lines express the OT gene and some were unresponsive to estradiol (Richard and Zingg, 1990, Burbach et al, 1990; Adan et al 1991). Therefore, in order to conduct these studies, the estradiol receptor (E_R) gene was cotransfected along with constructs containing the OT promoter region driving the reporter CAT (chloramphenicol acetyl transferase) gene. An imperfect (GGTG*ACCTTGACC) but fully functional estrogen response element (ERE) at position -164 and two additional sequence elements that correspond to the right half of the ERE (TGACC) were detected at positions -103 and -83. These half "ERE" did not impart any estradiol inducibility to the OT promoter and did not interact synergestically with the upstream functional ERE. The contextual sequences around the imperfect ERE are required to impart estradiol responsiveness as the putative ERE alone, cloned in front of TK-CAT, was unable to stimulate CAT activity in the MCF-7 cells (Mohr et al, 1989). This suggests the possibility of interaction of ER with other cis-acting elements or a requirement for a transcription factor which binds to the contextual sequences. It also stresses the importance of conducting these studies in cell lines that express the OT gene.

Despite the stimulatory effects of estradiol on circulating levels of OT *in vivo* and on OT promoter *in vitro,* the physiological significance of regulation of hypothalamic OT gene expression by estradiol remains controversial. Estradiol had no effect on hypothalamic OT mRNA levels in ovariectomized adult female rats (Burbach et al, 1990), but it increases OT mRNA in the preoptic area (Caldwell et al, 1989).

Figure I-4. Hormone responsive elements on the OT gene.

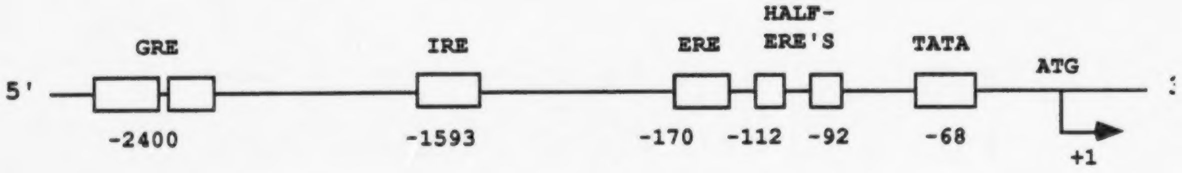
CONCENSUS ERE:

GGTCA nnn TGACC

OT GENE SEQUENCES:

	*	
-170	GGTGA CCT TGACC	-158
-112	GAACA GTT TGACC	-100
-92	CCTGG CTG TGACC	-80

OT GENE PROMOTER REGION:



ERE - estrogen responsive element
GRE - glucocorticoid responsive element
IRE - interferon responsive element

The lack of estradiol responsiveness may be due to an absence of estradiol receptors in the SON. Only a subpopulation of oxytocinergic cells in the PVN, which project centrally, contain estradiol receptors (Burbach et al, 1990; Rhodes et al, 1981; Rhodes et al, 1982). There are not enough *in vivo* studies in the literature to conclude the effects of estradiol on OT biosynthesis. The peripheral increase in circulating OT in response to estradiol may be due to increased release from the neurohypophysis. Estradiol effects on OT synthesis may be indirect through neurons which impinge upon oxytocinergic neurons. Further studies are required to understand the regulation of hypothalamic OT gene expression by estradiol.

The effects of progesterone on OT release and synthesis are not well understood. In female rats, both estrogen and progesterone increase the amount of OT present in the posterior lobe and gonadectomy reduces the amount slightly (Robert and Share, 1968,1970, Swaab and Jongkind, 1970; Jakubowska-Naziemblo, 1979; Amico et al, 1981a,b). Robert and Share (1968) also reported that administration of progesterone inhibits the increase in circulating OT following vaginal distention of the ewe. This difference in the effect of progesterone on OT release may be due to species variation or specific response to the stimulus used. In humans, a decrease in plasma levels of OT during the early luteal phase has been correlated with higher circulating progesterone levels (Shukovski et al, 1989) and this could be due to an inhibitory effect of progesterone on the hypothalamo-neurohypophyseal axis or to a lack of estradiol stimulation. It is not known whether progesterone has any effect on OT synthesis in the hypothalamus. At the cellular level, the effects of progesterone are mediated by the glucocorticoid response elements (GRE). The rat OT promoter contains two GRE at -2392 and -2354. The function of these is not known.

Thyroid hormone receptor (TR) is also a member of the steroid receptor superfamily and the thyroid response element (TRE) differs from the ERE by the absence of three central nucleotides. Thyroid hormone receptors are present in the hypothalamic nuclei expressing the OT gene and a decrease in OT biosynthesis is observed in thyroidectomized rats (Talanti and Attila, 1972). Thyroid hormones directly stimulate the activity of the rat and human OT promoters and the responsiveness is located in the same region as the ERE in both promoters (Adan et al, 1991). No cooperativity between ER and TR was observed when OT promoter was used.

The same region also mediates responsiveness to retinoic acid and a cooperativity between thyroid hormone, estradiol and retinoic acid is observed when P19 EC cells and human embryonal kidney 293 cells **were** used (Adan et al, 1992; Richards and Zingg, 1991). However, Lipkin et al (1992) demonstrated that the ERE of the rat OT promoter avidly binds the retinoic acid receptor (RAR) and functions as a negative retinoic acid response element in CV-1 cells (African green monkey kidney cell line). In view of these conflicting studies it is difficult to conclude the effects of retinoic acid on OT gene expression. Vitamin A deficiency or the treatment of neonatal rats with retinoic acid had no effect on the hypothalamic OT mRNA or peptide levels, suggesting that *in vivo*, retinoic acid does not influence OT gene expression.

3.5.6 Other potential regulators of OT

VP supresses OT release from the *in-vitro* superfused rat hypothalamic-pituitary complex (Miyake et al, 1983). The site of action or significance of this inhibitory influence *in vivo* is not known but this study cleary demonstrates that VP and OT interact with each other to influence their secretion.

Additionally, OT itself may play a role in positive feed back regulation of its own release (Moos et al, 1984; Inenaga and Yamashita, 1986; Falke, 1989; Moos and Richard, 1989). Injections of OT into the third ventricle has a strong facilitatory effect on the neurosecretory activation of OT neurons during suckling and this effect can be blocked by OT antagonists (Freund-Mercier and Richard, 1984).

Substance-P has been localized in the hypothalamas and pituitary and intracerebroventricular injections of this peptide increases the firing rate of magnocelluar neurons (Clarke et al, 1980; Cooper et al, 1981; Holtzbauer et al, 1984). The physiological significance of these is not known.

Both central and peripheral cholecystokinin octapeptide (CCK) and VIP systems are influenced by feeding (Bardrum et al, 1987). Peripheral administration of CCK is associated with an increase in OT release and electrical activity in a large proportion a putative OT cells in the SON (Verbalis et al, 1986; Renaud et al, 1987;

Blackburn and Leng, 1990). The pathways by which CCK influences the magnocelluar system are undefined, although gastric vagotomy severely blunts CCK-induced release of OT in conscious rats suggesting that gastric signals associated with food intake might be a physiological stimulus for OT release (Verbalis, 1986).

Inhibin and *activin* are gonadal glycoprotein hormones that are also synthesized in the brain and anterior pituitary (Meunier et al, 1988). Inhibin, first described because of its ability to inhibit the release of follicle stimulating hormone (FSH) (McCullagh et al,1932) is composed of two subunits, α and β, linked by disulfide bridges (Robertson et al, 1985; Miyamoto et al, 1985; Ling et al, 1985; Rivier et al, 1985). Two highly related forms of the smaller subunit, designated βA and βB, have been described (Vale et al, 1988). In contrast, activin consists of homo- and heterodimers of the inhibin β subunits and has been demonstrated to selectively stimulate FSH release (Ling et al, 1986). Recently, activin has been implicated in regulation of the neural oxytocinergic system. More specifically, a projection from the nucleus tractus solitarius to hypothalamic oxytocinergic neurons has been demonstrated to be immunoreactive for the activin βA chain and has been proposed to be important in OT biosynthesis and mediation of the milk ejection reflex (Sawchenko et al, 1988).

Similar to VP hyperosmolarity also stimulates OT release and synthesis (Jones and Pickering, 1969; Brimble and dyball, 1977; Mink et al, 1986; Balment et al, 1986; Cheng and North, 1986;

Dogterom et al, 1977; Balment et al, 1986; Savio et al, 1986; VanTol et al, 1987). The physiological significance of this is not known although OT has been suggested to play a role in electrolyte and water balance.

3.6 FUNCTIONS OF OT

The first described actions of OT are its contractile effects on the uterine myometrium and myoepithelial cells of the mammary gland. OT is also involved in a wide variety of reproductive activities ranging from regulation of the hypothalamo-pituitary-gonadal axis to a role in maternal behaviour. The studies for this thesis are focused on the role and regulation of OT during sexual maturation and parturition. Therefore, these two areas are discussed in more detail in section 4. Other central and peripheral functions of OT are briefly discussed below.

3.6.1 BEHAVIOUR

Centrally the most widely studied effects of OT are on maternal and sexual behaviour (section 4.3). Maternal behaviour in the rat consists of a number of integrated elements concerned with prolonged nurturing of large litters of pups (Elwood, 1983). This complex pattern appears spontaneously after parturition in inexperienced mothers. In the last few years the idea has been advanced that OT may play a role in the rapid induction of post-partum maternal behaviour. Pedersen and Prange (1979) were the first to attempt a systematic evaluation of a possible role of central OT on the induction of maternal behaviour. They were able to elicit full maternal responsiveness to foster pups in a significant proportion of virgin female rats by injection of OT into cerebral ventricles. Furthermore pup-induced maternal behaviour can be inhibited by intracerebroventricular (i.c.v) injections of anti-OT serum and OT antagonists (Pedersen and Prange, 1985; Fahrbach et al, 1985; van Leengood et al, 1986a). The centrally projecting parvocellular neurons are more important in induction of maternal behaviour (Caldwell et al, 1987; Insel and Harbaugh, 1989) .

The post partum increase in OT binding sites occurs selectively in the bed nucleus of stria terminalis, the region responsible for maternal behaviour and is dependent on estrogen priming (Insel, 1986; Kendrick et al, 1987). The relevance of these findings to humans is not known but OT appears to play an important role in the postpartum maternal behaviour of rats and sheep.

OT is also involved in modification of the brain mechanisms involved in conditioned avoidance behaviour and its effects are opposite to those induced by VP (DeWeid, 1979a,b; kovacs et al, 1978, 1982). OT has also been demonstrated to attenuate memory consolidation and retrieval processes in the laboratory animals (Kovacs and Telegdy, 1982). OT, its six-membered ring structure metabolite (tocinoic acid and its amide) and MIF-1 (the C-terminal tripeptide OT metabolite) facilitate avoidance reactions in rats suggesting that OT may possess more than one behaviourally active core able to influence learning and memory processes (DeWeid 1979; Kovacs et al, 1983, 1985). In rats, OT is also associated with learned taste aversion (Blackburn and Leng,1990).

3.6.2 Lactation

The involvement of OT in milk ejection was postulated by Ely and Peterson in 1941. In various animal species transient increases in blood levels of OT occur during milking or suckling (Cleverly and Folley, 1970; Johnson and Amico, 1986). The afferent inputs for OT release are relatively poorly characterized. Stimulation of the nipple, presence of babies and other factors associated with the routine of nursing or milking play a role in milk ejection (Lincoln and Wakaley, 1975). The nipple and skin covering the mammary gland have a rich supply of sensitive nerve endings that responds to touch and pressure and is involved in reflex milk ejection (Sala et al, 1974). On the other hand, denervated transplanted goat udders can maintain normal milk output (Linzell, 1963). The relative importance of individual stimuli in milk ejection depends on species.

4. OT DURING SEXUAL MATURATION IN THE RAT

4.1 PUBERTAL DEVELOPMENT

Puberty is the developmental transition between the juvenile and adult reproductive states, during which physical and behavioural status changes profoundly. Sexual development of the rat can be devided into four phases (Critchlow et al, 1967; Ojeda et al, 1980; 1984). The *neonatal* period, which comprises the first week after birth; the *infantile* period which extends from days 8-21; the *juvenile* period which ends around day 30-35; and finally the *peripubertal* period which ends at about 45-55 days of age in the male rat while in female rats it culminates with the occurrence of first ovulation, about day 38-40.

Circulating levels of gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH), sex steroids and prolactin change during pubertal maturation (Ojeda and Ramirez, 1972; Ojeda et al, 1976; Ojeda and Ramirez, 1974; Ojeda et al, 1975; Steele et al, 1977) and play an integral role in onset of puberty. In female rats the timing of the first ovulatory surge of LH depends on the completion of ovarian maturation. It has been shown that OT biosynthesis increases during pubertal maturation in both male and female rats (Jackson and George, 1980; Miller et al 1989). The function of OT during pubertal maturation is not known. OT is implicated in many central and peripheral reproductive functions. Local increases and cyclic changes in OT biosynthesis in the central nervous system may modulate sexual behaviour in a paracrine fashion. Elevation of OT levels in the circulating plasma at the time of pubertal maturation may directly or indirectly affect steroidogenesis and ovarian function, or facilitate motility of oviducts and sperm transport thereby coordinating central and peripheral reproductive functions.

Prolactin (Prl) also plays a role in the pubertal maturation of the rat (Advis and Ojeda, 1978; Advis et al, 1981a,b; 1982). The levels of Prl are low at the beginning of the juvenile period but increases thereafter and reach peak levels at puberty. Intrahypothalamic or systemic administration of prolactin accelerates pubertal development in female and male rats, partly by increasing the number of LH receptors in the ovary. OT also stimulates prolactin release (Lumpkin et al, 1983; Antoni, 1987; Johnston and Negro-Vilar, 1988; Mori et al, 1990). Both increase around pubertal maturation, play a role in sexual behaviour of rats and their circulating levels increase in response to estradiol. The promoter regions of both the OT and prolactin genes contain estradiol responsive elements and the transcription of the genes increases in response to estradiol. However, increased prolactin secretion during pubertal maturation is partly independent of the gonads, (Kimura and Kawakami, 1981), suggesting maturation of neurons or a change in central drive occurs as the female rat matures.

The mechanism by which the OT gene is regulated during sexual maturation is not known. The effects of estradiol on OT biosynthesis in adult rats are controversial. Therefore one of the objective of this thesis was to determine whether OT biosynthesis is regulated by estradiol during pubertal maturation.

4.2 OT During the Estrous Cycle

The rat is a nonseasonal, spontaneously ovulating polyestrous mammal. The rat estrous cycle occurs every 4-5 days and is divided into four phases depending upon changes in hormones, uterine tract and behaviour. The first part of the estrous cycle is termed *proestrus*, the time when the animal is beginning ovarian hormonal secretion and follicular maturation. The next stage, *estrus* is the time when the female is sexually receptive. Estrus is followed by *metestrus*, during which the estrous changes in the reproductive tract subside. *Diestrus* is of variable duration in different species. During this period the ovarian secretions prepare the reproductive tract for the receipt of fertilized ova. If fertilization has not taken place, the mammal returns to proestrus and the cycle begins anew.

Most of our knowledge concerning the hormonal events during the estrous cycle (and the menstrual cycle in primates) has evolved from measurements of hormones in plasma. Gonadotropins, estradiol, progesterone, prolactin and OT are all integral part of the estrous cycle and together coordinate various reproductive functions. The cyclic release of LH, FSH and prolactin is the result of positive and negative feedback control by ovarian steroids acting at both the hypothalamus and pituitary gland (Dohler and Wuttke, 1974; Meijs-Roelof et al, 1975). In intact rats, circulating estradiol concentrations begin to rise early in diestrus and peak 24-48 hours later in proestrus (Docke et al, 1984; Dohler and Wuttke et al, 1974). Ovarian estradiol secreted on diestrus is the positive-feedback stimulus for the ovulation-inducing surge of LH as well as for the proestrus surge of prolactin. On the afternoon of proestrus, the circulating levels of LH begin to increase rapidly and reach peak levels in the evening. This rapid surge of LH induces follicular rupture and ovulation (Butcher et al, 1974).

Several studies have found that the OT content of the posterior pituitary, as well as plasma levels, vary during the estrous cycle and vary in response to circulating estrogen and progesterone concentrations (Heller, 1958; Konig and Ehlers 1973; Negoro et al, 1973; Crowley et al, 1978; Pitzel et al 1981). In parallel to plasma estradiol levels, maximal OT concentrations are observed at proestrus and are lowest at metestrus (Crowley et al 1978; Sarkar, 1984) while OT levels were highest in both the SON and PVN at diestrus and lowest at proestrus, when estradiol levels peak indicating that OT is transported out of the cell bodies into the neurohypophysis (Greer et al, 1986). The proestrous accumulation of OT may serve as a pool for OT release into the portal circulation on the afternoon of proestrous.

The mid-cycle increase in estradiol stimulates OT release but its effect on OT synthesis is not known. To answer this question van Tol et al (1988) examined changes in OT gene expression in the microdissected SON (primarily responsible for neurohypophyseal OT content and therefore for OT in the peripheral circulation) during the estrous cycle. In the SON, OT mRNA levels were highest (1.5 fold increase) at estrus and OT peptide levels in the pituiary gland were lowest at this stage, suggesting secretion of OT into the circulation. Only the SON was examined in this study and may not be representative of total OT synthesis in the hypothalamus. Furthermore, estradiol concentrating cells are mainly present in the PVN rather than the SON. Therefore, the increase in OT mRNA in the SON during estrus may be an indirect effect of estradiol, or the estradiol effect may occur in a non-receptor-mediated fashion.

These studies indicate a correlation between OT and estradiol but do not prove a cause and effect relationship. Increased estradiol levels may not be necessary for increased OT gene expression (van Tol et al, 1988; Zingg et al, 1988). More studies are required to elucidate the nature of the relationship between estradiol and OT biosynthesis and secretion during the estrous cycle.

These changes in OT are not limited to rats. Circulating concentrations of OT and it[illegible] associated neurophysin are correlated with the mid-cycle surge of LH and with elevated estrogen levels in humans and non-human primates (Legros et al, 1975, Kumaresan et al

1983, Robinson et al 1976, 1977). The increase in plasma levels of OT may be pituitary or gonadal in origin. However, in agonadal and postmenopausal women serum immunoreactive OT increased in response to exogenously administered estradiol (Shukovski et al, 1989), suggesting circulating OT is mainly pituitary in origin.

Several central and peripheral roles for OT during the estrous/menstrual cycle have been proposed (Robinson and Evans, 1990). Amico et al (1989) have suggested that one possible function of OT during ovulation is to stimulate the release of LH from the pituitary (Evans and Catt, 1987). However, studies of the interactions between OT and pituitary gonadotropin release are conflicting. An increase in both FSH and LH in plasma and urine occurs in response to OT injection (Martini et al, 1959). In contrast, other investigators (Ditlove and Faiman 1970; Dawood et al, 1980) did not observe any changes in gonadotropin levels in response to OT treatment, either with or without estrogen pretreatment. Another role of OT during estrous cycle is to modulate sexual behaviour and is discussed below.

4.3 Sexual Behaviour

OT is one of the centrally active neurotransmitters known to influence reproductive behaviour in rats (Caldwell et al, 1986., Arletti et al, 1985; Gorzalka and Lester, 1987; Hughes et al, 1987). The facilitative effects of OT on sexual receptivity are dependent on ovarian hormone priming (Gorzalka and Lester, 1987).

The biochemical process stimulated by estradiol relevant to OT effect is the induction of central OT receptors in certain brain regions that contain estradiol concentrating cells (Rhodes et al 1981). In particular, the VMN (area responsible for sexual behaviour) shows a marked increase in OT binding sites in response to estradiol and testosterone treatment in both male and female rats (Dekloet et al, 1986; Corini et al 1989). Treatment with estradiol also increases OT immunoreactivity in the fibres which project from the rostral part of the PVN to the VMN (Johnson et al, 1989a,b), suggesting estradiol has dual functions: 1) it increases OT release centrally, and 2) it increases target responsiveness by inducing OT receptors. In intact rats, circulating estradiol concentrations begin to rise early in diestrus and peak 24-48 hours later in proestrus (Docke et al, 1984; Dohler and Wuttke et al, 1974). Maximum sexual receptivity occurs approximately 4h after the estradiol peak. Thus, estradiol dependent changes in OT and its receptors occur within a similar time frame to fluctuations in sexual responsiveness during the estrous cycle.

Similar to OT, prolactin has been implicated in many reproductive functions and sexual behaviour. In female rats, a marked estradiol-dependent prolactin surge begins during proestrus and peaks during estrus (Johnston and Negro Vilar, 1988). A similar midcycle surge in prolactin levels occurs in women. As already discussed, OT is a prolactin releasing factor and some of the *in vivo* effects of OT may be mediated by prolactin. A positive feedback loop between prolactin and OT has been suggested by Sarkar

(1989), who noted that, especially during lactation, each can propagate the other's effects.

The peripheral actions of OT include stimulation of uterine and oviduct smooth muscle motility, to promote fertilization by enhancing sperm and ova transport after mating. Several studies have demonstrated an increase in circulating levels of OT during sexual activity in rats and humans (Cramichael, 1987; Caldwell et al, 1988). The role of peripheral OT during mating remains controversial. Studies in bulls showed no release of OT during mating and ejaculation, whereas mechanical or electrical stimulation of the genitalia causes a substantial release (Schams et al, 1982; Peeters et al 1983). Recently, Arletti et al (1990) investigated male sexual behaviour in sexually normal and sexually sluggish 20 month old rats. These studies demonstrated that OT plays a facilitatory role in mating behaviour. Mating also induces a bimodal pattern of prolactin secretion in female rats and this pattern is partially regulated by OT.

5. SUMMARY AND HYPOTHESES

The preceding literature review suggests that OT is an important reproductive hormone. Increases in OT biosynthesis and release around the time of pubertal maturation may play a significant role, along with estradiol, in coordinating various ovarian, sexual and behavioural functions. This increase in OT biosynthesis may be the result of increasing circulating levels of estradiol due to ovarian maturation. However the possibility that an increase in OT gene expression during pubertal maturation is independent of gonadal steroids cannot be excluded. For example, activin also may stimulate increased synthesis of OT in the hypothalamus. To understand the role of gonadal steroids and/or factors in the regulation of OT gene expression during pubertal maturation in both male and female rats we hypothesized that:

1. **Increased circulating levels of gonadal steroids during pubertal maturation, both in male and female rats, increases OT gene expression.**

2. **Changes in circulating estradiol concentrations affect OT gene expression during the estrous cycle.**

3. **Administration of estradiol/testosterone to infantile rats prematurely stimulates OT gene expression.**
4. **Activin biosynthesis increases during puberty and thus may increase OT gene expression.**

These hypotheses were examined and the results are discussed in the accompanying paper published in the December 1990 issue of *Molecular Endocrinology*.

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CHAPTER II

REGULATION OF NEURAL OXYTOCIN GENE EXPRESSION BY GONADAL STEROIDS IN PUBERTAL RATS

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INTRODUCTION

The nonapeptide oxytocin has been implicated as a neurosecretory peptide involved in labor and milk ejection and as a centrally active neuropeptide responsible for stimulating sexual and maternal behaviors (2-5). Oxytocinergic neurons in the supraoptic and paraventricular nuclei of the hypothalamus project to the posterior pituitary where the peptide is released into the circulation (6). In addition, oxytocinergic parvocellular neurons have been shown to project to many extrahypothalamic brain areas, including regions of spinal cord, brainstem, and forebrain (6,7). Gonadal steroids, which play a central role in the control of reproductive function, influence both neurosecretory and intracerebrally projecting oxytocinergic neurons. Estrogen treatment stimulates the release of oxytocin into the peripheral circulation (8), induces oxytocin binding sites in the brain (9), influences the electrical activity of oxytocinergic neurons (10), and alters the pattern of oxytocinergic cells and fibers in the brain, as detected by immunocytochemistry (11-13). How estrogen mediates these changes is unknown, although a subpopulation of oxytocinergic neurons have binding sites for estradiol (14,15), and the oxytocin promoter region is capable of conferring estradiol-sensitivity on a heterologous reporter gene in cells expressing the estradiol receptor (16).

Inhibin and activin are gonadal glycoprotein hormones that are also synthesized in the brain and anterior pituitary (17). Inhibin, first described because of its ability to inhibit the release of follicle-stimulating hormone (FSH) (18) is constituted of two subunits, a and ß, linked by disulfide bridges (19-22). Two highly related forms of the smaller subunit, designated ßA and ßB, have been described (21). In contrast, activin consists of homo- and heterodimers of the inhibin ß chains and has been demonstrated to selectively stimulate FSH release (23, 24). Recently, activin has been implicated in regulation of the neural oxytocinergic system. More specifically, a projection from the nucleus tractus solitarius to hypothalamic oxytocinergic neurons has been demonstrated to be immunoreactive for the activin ßA chain and has been proposed to be important in oxytocin biosynthesis and mediation of the milk ejection reflex (25).

Puberty is the developmental transition between the juvenile and adult state, during which the hormonal, physical, and behavioral status of an animal changes profoundly. Factors that control the onset of puberty are incompletely understood but involve a reactivation of the hypothalamo-pituitary gonadotropin-gonadal apparatus (26). The increased secretion of gonadal steroids, which coincides with this reactivation, subsequently influences the development of reproductive function and behavior. In both male and female rats, the levels of endogenous circulating estradiol start to rise on postnatal day 33, and continue to increase until around postnatal day 40 (27,28). In males there is also a corresponding increase in testosterone over the same timeframe (27).

We have previously demonstrated that neuronal oxytocin mRNA levels increased during pubertal development of female rats (1). Increased mRNA was detected in oxytocinergic neurons throughout the hypothalamus, including those known to project as magnocellular neurons to the neurohypophysis and those of parvocellular origin thought to make wholly intracerebral connections. In the studies reported here, we have extended these findings, and have demonstrated that the pubertal increase in neural oxytocin mRNA occurs in both male and female rats and is largely dependent upon circulating gonadal steroids, but is not correlated with changes in neural inhibin/activin ßA chain mRNA. Regulation of oxytocin mRNA by gonadal steroids during puberty provides a physiological mechanism for coordinating the peripheral and central effects of this peptide on the development of reproductive functions.

MATERIALS AND METHODS

ANIMALS AND SURGICAL PROCEDURES

Sprague-Dawley rats were maintained at 22° C with free access to food and water in a 12:12 hour light and dark cycle. Animals were sacrificed under deep pentobarbital anaesthesia (35 mg/kg), and the brains removed and frozen on dry ice prior to dissection. Frozen brains were dissected to produce three different brain regions. The forebrain sample included all tissue rostral to the anterior commissure, as well as the cerebral cortex. The middle brain segment included tissue from the rostral border of the anterior commissure extending caudally to the caudal border of the tuber cinereum. This fraction contained all previously-described oxytocinergic neurons. All tissue caudal to the tuber cinereum was included in the brainstem sample. Oxytocin mRNA was only detected in RNA isolated from the middle brain segment samples.

Developmental studies: Male and female Sprague-Dawley rats at postnatal days 20 (P20), 40, and 60 (6 each) were sacrificed under deep pentobarbital anaesthesia. For mature, cycling female rats, the estrous cycle stage was determined by vaginal cytology, and 4 rats each were sacrificed at proestrous, estrous, metestrous, and diestrous.

Steroid replacement studies in pubertal animals: Sprague-Dawley rats were weaned at P20 and male and female rats were subsequently maintained separately. Bilateral gonadectomies were performed via ventral midline incision under metophane anaesthesia. Steroid-filled silastic tubing (Polydimethylsiloxane, i.d. 1.57 mm and o.d. 3.15 mm, Dow Corning Co., Midland, MI) was subsequently implanted subcutaneously (medial dorsal). The silastic implants were made according to the method of Legan et al (29) except that they were incubated overnight in sterile water to minimize the transitory increase in circulating steroids. Animals were divided into four groups of 2 male and 2 female rats each. The sham-operated control and gonadectomized rats received empty silastic implants. In the treatment groups, rats received silastic implants containing estradiol (0.5 cm) or testosterone (2.5 cm) (Sigma). This length of implant has been demonstrated to maintain steroid levels within the physiological range (30,31). Rats were sacrificed at P60 under deep pentobarbital anaesthesia. The replacement experiments were repeated 3 times.

Steroid replacement studies in prepubertal animals: Intact P10 male and female rats (2 animals of each sex in 3 different groups) received silastic implants containing estradiol (0.3 cm) or testosterone (1.2 cm) subcutaneously under metophane anaesthesia. The control animals received empty silastic implants. Rats were sacrificed at P20 under deep pentobarbital anaesthesia (35 mg/kg). The prepubertal replacement experiments were repeated twice.

STEROID DETERMINATIONS

Plasma estradiol and testosterone concentrations were measured by specific radioimmunoassay kits purchased from Diagnostic Systems Laboratories Inc. (Webster, TX.). Plasma samples were obtained at the time of sacrifice and duplicate samples from individual animals were measured. For estradiol, the minimum detectable concentration was 110 pmol/L and for testosterone was 0.35 nmol/L. For both assays, the intraassay and interassay coefficients of variation were <9%.

RNA ISOLATION AND ANALYSIS

Total cytoplasmic RNA (1-5 ug) was prepared and analyzed by Northern blot analysis as previously described (1,32). Nitrocellulose filters were subsequently exposed to XAR or XRP X-ray film (Kodak). Recognizing that Northern blot analysis is a semi-quantitative technique, we attempted to maximize accuracy and precision by confirming that equal amounts of RNA were loaded in each lane. To this end, ethidium bromide was added to the sample buffer prior to electrophoresis, and gels were photographed under ultraviolet illumination. In addition, the nitrocellulose was stained with methylene blue (33) subsequent to hybridization.

Probes specific for oxytocin mRNA were prepared as previously described (1) from a genomic clone kindly provided by Dr. Hartwig Schmale (34). Antisense RNA probes specific to the ßA subunit of inhibin/activin were generated from a subclone kindly provided by

Dr. Helene Meunier (17) under conditions described by Melton et al. (35).

Northern blot results were quantitated using an LKB Ultrascan XL scanning laser densitometer. To make quantitative comparisons, Northern blots from different experiments were chosen after ensuring that the amounts of total RNA in the pertinent lanes were identical. Several different film exposures of the same data were analyzed to obtain films with exposures that were optimal for densitometry. Results are represented as an approximate value, or as a range of values.

RESULTS

To determine whether neural oxytocin mRNA increases in pubertal male rats as it does in females, we isolated total middle brain segment RNA from both sexes prior to and subsequent to puberty. Northern blot analysis demonstrated that oxytocin mRNA levels increased 5 to 10-fold following postnatal day 20 to attain adult levels by postnatal day 60 in both male and female rats (Fig. 1a, 1b). Oxytocin mRNA levels at P40 were intermediate between those detected at P20 and P60 (data not shown). Thus, oxytocin mRNA increases during puberty to a similar degree in both sexes.

We previously demonstrated that ovariectomy of mature female rats decreased levels of neuronal oxytocin mRNA, suggesting that gonadal steroids regulate oxytocin gene expression (1). To determine whether physiological variations in the levels of circulating plasma estradiol influenced neural oxytocin mRNA in mature animals, we isolated total RNA from female rats at proestrous, estrous, and diestrous. Northern blot analysis indicated that levels of oxytocin mRNA were similar at all stages of the estrous cycle (Fig. 1c). Furthermore, levels of oxytocin mRNA in cycling female rats were similar to levels in mature males (Fig. 1d).

To determine whether the observed pubertal increases in oxytocin mRNA were due to maturation of the gonads, or to other changes associated with puberty, we performed prepubescent gonadectomies of male and female rats at P20 and isolated total middle brain segment RNA at P60. Northern blot analysis demonstrated that pubertal upregulation of oxytocin mRNA was largely, but not completely, inhibited by gonadectomy (Fig. 2). In both male and female rats, the pubertal increase was approximately 2-fold in ovariectomized animals versus 5 to 10-fold in sham-operated control animals. Thus, maturation of the gonads is partially responsible for the developmental increase in oxytocin mRNA, but other as-yet-undefined factors also play a role.

To determine whether estradiol and/or testosterone are the gonadally-derived "factors" responsible for the pubertal increase in oxytocin mRNA, female and male rats were gonadectomized at P20, implanted with steroid-filled silastic tubing, and sacrificed at P60. To ensure that the implants were effective, circulating plasma levels of both steroids were determined in control and implanted animals at P60. Following castration, plasma estradiol levels were non-detectable in our assay (< 110 pmol/L). After replacement with estradiole silastic tubing, concentrations reached 789 ± 215 pmol/L (mean ± standard deviation). By comparison, estradiol concentrations are approximately 400 to 500 pmol/L on the day of estrous in normal rats (27, 28). Castrate levels of testosterone also were non-detectable (<0.35 nmol/L) and were 9.0 ±

6.6 nmol/L following silastic tubing replacement. This compares with normal rat testosterone concentrations of 3.5 to 7.0 nmol/L (30). Interestingly, in the testosterone replaced rats, plasma estradiol levels were 853 ± 267 pmol/L, presumably due to peripheral aromatization. Northern blot analysis of total RNA isolated from the middle brain segment of these implanted animals demonstrated that estradiol or testosterone treatment of gonadectomized animals of both sexes increased oxytocin mRNA to levels similar to adult control animals (Fig. 3).

EXPRESSION OF NEURAL ACTIVIN ßA CHAIN mRNA

Circulating steroids could affect neural expression of activin, thereby indirectly regulating the hypothalamic oxytocinergic system. To address this possibility, we determined levels of inhibin/activin ßA chain mRNA in the nervous system following gonadectomy and steroid replacement (Fig. 4). Northern blot analysis of total RNA demonstrated that ßA chain mRNA in the brain is approximately 6.8 kb (Fig. 4), as previously reported for this mRNA in rat ovary (36). Levels of ßA chain mRNA appeared to be similar in the cortex and brainstem, and somewhat lower in the middle brain segment (Fig. 4d). The abundance of ßA chain mRNA in the middle brain segment of male and female rats appeared not to be affected by either prepubescent gonadectomy or by steroid treatment (Fig. 4a, b, and c). ßA chain mRNA levels in the brainstem segment, which contains any ßA chain mRNA synthesized in the nucleus tractus solitarius, also appeared unaffected (Fig. 4e) Thus, it appears unlikely that steroid hormones act via an activin intermediate to regulate neural oxytocin mRNA levels during puberty although it remains possible that activin may be regulated in a specific subset of cells and the total change is too small to be measured by this semi-quantitative approach.

One possible explanation for low levels of oxytocin mRNA at P20 is limited availability of prepubertal steroids. To test this hypothesis, steroid-containing silastic implants were placed subcutaneously in intact, postnatal day 10 male and female rats. Animals were subsequently sacrificed at postnatal day 20, and total RNA was isolated from the middle brain segment (Fig. 5). Although steroid estimations were not done for these animals, the increased size and vascularity of the reproductive organs of experimental animals demonstrated that steroid levels were significantly higher than those of controls. Northern blot analysis revealed that neither testosterone nor estradiol were able to increase prepubescent levels of neural oxytocin mRNA (Fig. 5), suggesting that oxytocinergic neurons do not yet express the appropriate receptors and/or that neural maturation (which is not completed until approximately P20) is a necessary requisite for steroid sensitivity.

DISCUSSION

Results of this study demonstrate that, concomitant with puberty, neuronal oxytocin mRNA levels increase 5 to 10-fold in male, as well as female, rats. This increase is dependent both upon the presence of intact gonads, and upon other, as-yet-undefined factors. Pubertal treatment of gonadectomized animals with either estradiol or testosterone is sufficient to induce the normal developmental increase in neural oxytocin mRNA. It is unlikely that the observed effects of steroid hormones are mediated indirectly via neural activin, since ßA chain mRNA levels appeared to be unaffected by gonadectomy or steroid treatment. Interestingly, treatment of prepubescent animals with either of these steroids is insufficient to prematurely increase oxytocin mRNA levels, indicating that neuronal maturation may be a prerequisite for the observed steroid sensitivity. Together, these data demonstrate that gonadal steroids play an integral role in the pubertal development of the oxytocinergic system.

Peripherally, oxytocin is important for both lactation and parturition (37,38) in females, while centrally, oxytocin is known to elicit sexual and maternal behavior (2,39,40,41). Oxytocin is synthesized both in the central nervous system, and in the ovary (42,43), where it is produced cyclically in the corpus luteum (44), and is believed to play an endocrine role (45). Furthermore, oxytocin may directly or indirectly affect pubertal maturation; oxytocin is believed to stimulate release of prolactin from the anterior pituitary (46,47), and hyperprolactinemia can enhance the onset of puberty (48). Oxytocin may therefore be important both during the cascade of events that cause puberty, and in the subsequent regulation of reproductive behavior and function in sexually-mature animals.

The functional maturation of the gonads during puberty leads to increased circulating levels of both estrogen and androgens (27,28). Our results indicate that induction of neural oxytocin mRNA during puberty is largely dependent upon the presence of these gonadal steroids. This relationship between increased gonadal steroids and increased neural oxytocin mRNA may be an essential component of the behavioral effects of steroids. Furthermore, since gonadal steroids are known to have broad effects on the central nervous system (49), it is possible that the oxytocinergic system is just one of several neural systems that undergo their final maturation under the influence of increased pubertal steroids.

Although gonadal steroids are required for the normal pubertal increase in oxytocin mRNA, our results also indicate that a significant component of the observed increase was independent of gonadectomy. Several direct and indirect mechanisms can be invoked to explain this observation. In addition to the gonads, the adrenal gland is a source of steroid hormones, and adrenally-derived steroids have been suggested to play a role in the onset of puberty (50,51). However, following castration, estradiol and testosterone levels were non-detectable in our experiments and it therefore seems unlikely that adrenal steroids play a physiologic role in the regulation of hypothalamic oxytocin gene expression. Insulin-like growth factor-1 (IGF-1) increases during puberty (52), and directly increases oxytocin mRNA in granulosa cells from the rat ovary (53). Alternatively, the gonad-independent increase could reflect an intrinsic, developmentally-programmed maturation of oxytocinergic neurons themselves, or of other neural systems that impinge on the oxytocinergic system.

Levels of oxytocin mRNA in gonadectomized animals following estradiol or testosterone replacement to either sex were similar to controls. These data may indicate that testosterone is mediating its effects on the oxytocinergic system via its aromatization to estradiol (54). Aromatization could occur in peripheral tissues, or within the central nervous system itself, where aromatase activity is highest in those regions associated with reproductive functions, such as the hypothalamus and limbic structures (55). However, testosterone also has effects that are independent of its aromatization; for example, intracerebral implants of dihydrotestosterone (a non-aromatizable, potent metabolite of testosterone) in castrated male rats was sufficient to activate sexual behavior (56,57). Thus, it is possible that receptors for estradiol and testosterone are colocalized on the appropriate target neurons, resulting in convergent regulation of oxytocin gene expression.

Gonadal steroids may act directly on oxytocinergic neurons themselves, and/or may indirectly influence oxytocin gene expression via other, steroid-sensitive neuronal populations. In support of a potential direct mechanism, the oxytocin promoter contains estradiol response elements (34), and is capable of conferring estrogen sensitivity on heterologous reporter gene constructs when transfected into tissue culture cells that express the estrogen receptor (16). Furthermore, in agonadal and postmenopausal women serum immunoreactive oxytocin increased in response to exogenously administered estradiol (43). Specific increases in oxytocin mRNA levels in the bovine ovary at the time of ovulation may be a function of increased estradiol (44) . However, only a subset of oxytocinergic neurons at sexual maturity bind radiolabelled estradiol (15), or express estrogen-receptor-like immunoreactivity (58), whereas all oxytocinergic neurons express increased levels of oxytocin mRNA following puberty (1). Thus, either some of the observed effects are indirect, estradiol is effecting oxytocin gene expression in a non-receptor mediated fashion, or most oxytocinergic neurons express the estrogen receptor during a restricted developmental period that includes puberty.

Although gonadal steroids induce neural oxytocin mRNA during puberty, the results presented here indicate that they are incapable of doing so prepubescently. Other studies demonstrate that treatment of infantile female rats from P10 to P26 with estradiol does not enhance puberty (59-63). Furthermore, endogenous circulating levels of estradiol are relatively high at birth, and surge again from P9 to P21 (27) without a concomitant high level of oxytocin mRNA expression (1, F.M. unpublished observations). The most likely interpretation of these data is that neural maturation is a prerequisite for the cascade of events that accompany puberty, including steroid sensitivity of the oxytocinergic system. The requisite maturation may include the developmental elaboration of steroid receptors on target neurons and/or establishment of the appropriate neural connections.

Following puberty, levels of neural oxytocin mRNA are similar in male and female rats. In agreement with data obtained by Ivell et al. (44), we also observed little or no difference in the levels of neural oxytocin mRNA during the estrous cycle. However, ovariectomy of mature female rats decreased neural oxytocin mRNA, consistent with gonadal regulation of this gene in mature animals (1). Together, these data indicate that at least some oxytocinergic neurons remain sensitive to circulating steroids in the mature animal, but that the lowest normal physiological levels (eg. during diestrous) are sufficient to maintain mature amounts of oxytocin mRNA. These data do not imply that neural oxytocin mRNA cannot be increased by a change in physiological status, or by other extrinsic factors. In fact, oxytocin mRNA can be regulated as a function of salt-loading (64), and increases during late pregnancy and lactation (1,65). The latter increase may also involve steroid hormones, and could be attributed either to increased levels of circulating steroids (66) or to modulation of the number of steroid receptors on oxytocinergic neurons (67). Alternatively, oxytocinergic neurons undergo significant morphological remodelling during pregnancy (68), which may lead to a concomitant intrinsic increase in expression of the oxytocin gene.

Although neural activin has been implicated in regulation of the oxytocinergic system, it is unlikely that it regulates any of the changes in oxytocin gene expression described in this study. Northern blot analysis demonstrated that inhibin/activin ßA chain mRNA is expressed at similar levels in the forebrain and the brainstem, with somewhat lower levels in the middle brain segment, indicating that synthesis of this hormone is more widespread than previously demonstrated by immunocytochemistry (25). Furthermore, levels of ßA chain mRNA in the middle brain segment and brainstem appeared unaffected by gonadectomy or steroid treatment, and are therefore unlikely to be associated with sexual maturation in either sex. However, we cannot rule out the possibility that significant changes occur in activin mRNA in a small subset of cells that influence oxytocin gene expression but escape detection by Northern blot analysis. Interestingly, activin has been recently reported to support survival of certain neuronal populations (69), suggesting that it may have functions in the central nervous system independent of the oxytocinergic system.

In summary, we have demonstrated that estradiol and testosterone regulate neural oxytocin gene expression during a developmental period that encompasses puberty. Gonadal regulation of this neuropeptide may provide a means of coordinately regulating the central and peripheral effects of this nonapeptide on the development and maintenance of the reproductive biology of both male and female rats.

FIGURE LEGENDS

Figure 1. Northern blot analysis of neural oxytocin mRNA from the middle segment of the brain in developing and mature male and female rats. The upper panels are photographs of autoradiographs produced by hybridizing Northern blots with a radiolabeled oxytocin riboprobe. The lower panels are photographs of the same blots stained with methylene blue following hybridization to demonstrate that equivalent amounts of total RNA were loaded in each lane. (a) RNA from male rats at P20 (lane 1) and P60 (lanes 2 and 3). (b) RNA from female rats at P20 (lanes 1 and 2) and P60 (lane 3). (c) RNA from mature female rats at proestrous (lane 1), estrous (lane 2) and diestrous (lane 3). (d) RNA from a mature female rat at proestrous (lane 1) and mature male rats (lanes 2 and 3). Note that in the lower panel of (d), all lanes were from the same photograph, but the methylene blue background was variable across the nitrocellulose filter.

Figure II-1.

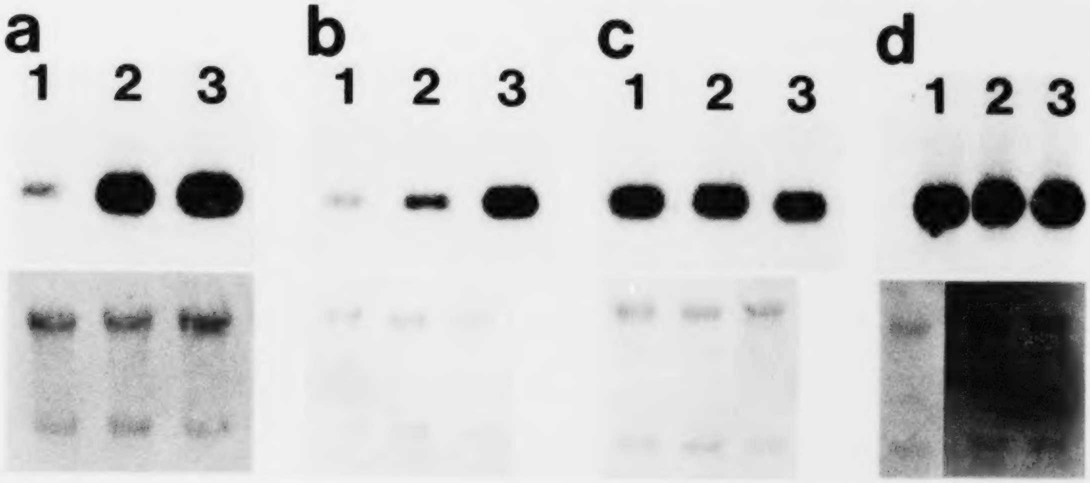


Figure 2. Northern blot analysis of oxytocin mRNA from the middle brain segment in (a) male and (b) female rats. Upper and lower panels are as described in Fig. 1. Lanes 1 and 2 contain RNA from P20 and P60 intact rats respectively. Lane 3 contains RNA from P60 rats that were gonadectomized on P20.

Figure II-2.

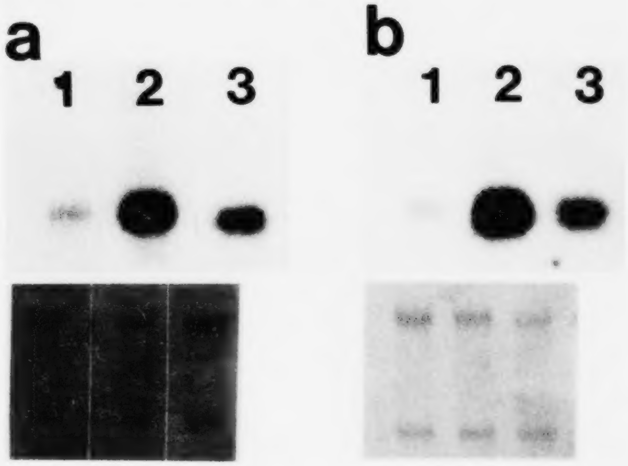


Figure 3. Northern blot analysis of oxytocin mRNA from the middle brain segment in (a) male and (b) female rats following prepubescent gonadectomy and sex steroid replacement. Upper and lower panels are as described in Fig. 1. (a) RNA from intact control P60 males (lane 1), P60 males gonadectomized at P20 (lanes 2 and 3) and P60 males gonadectomized at P20 and replaced with estradiol (lane 4) or testosterone (lane 5). (b) RNA from intact control P60 females (lane 1), P60 females gonadectomized at P20 (lane 2), and P60 females gonadectomized at P20 and replaced with estradiol (lane 3) or testosterone (lane 4).

Figure II-3.

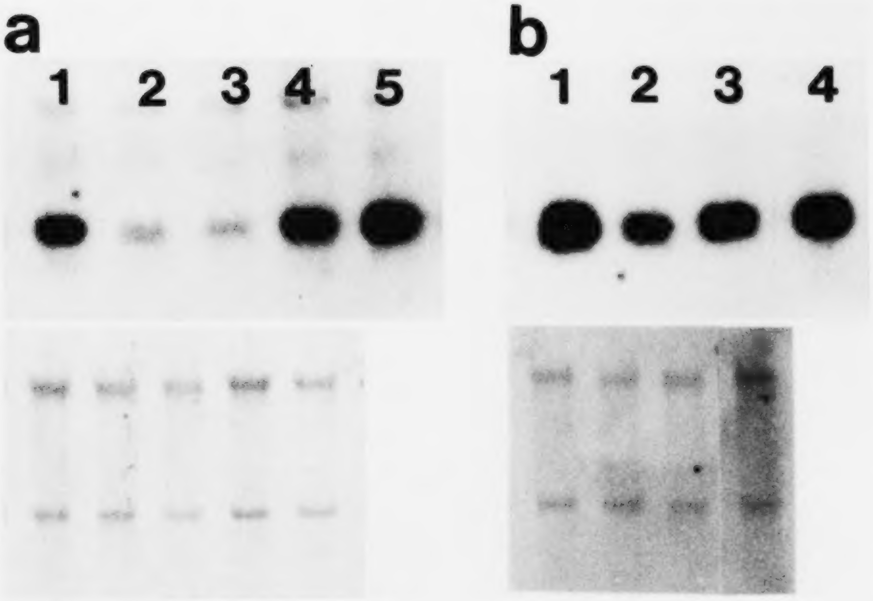


Figure 4. Northern blot analysis of inhibin/activin ßA chain mRNA in P60 female (panels a,b,d,e) and male (panel c) rats following gonadectomy at P20 and sex steroid replacement. Equal amounts of total RNA were loaded in each lane of any panel. The closed arrows denote the ßA chain transcript and the open arrows indicate the 28S and 18S ribosomal RNAs . (a) RNA from the middle brain segment of P60 intact control females (lane 1) and P60 females gonadectomized at P20 and replaced with estradiol (lane 2) or testosterone (lane 3). (b) RNA from the middle brain segment of P60 intact control females (lane 1) and P60 females gonadectomized on P20 (lane 2). (c) RNA from the middle brain segment of P60 intact control males (lane 1), P60 males gonadectomized at P20 (lane 2), P60 males gonadectomized at P20 and replaced with estradiol (lane 3) or testosterone (lane 4). (d) RNA from P60 intact control females, extracted from cerebral cortex (lane 1), middle brain segment (lane 2) and brainstem (lane 3). (e) RNA from the brainstem of P60 female rats gonadectomized at P20 (lane 1) or gonadectomized at P20 and replaced with estradiol (lane 2) or testosterone (lane 3). Note that the increased signal for ßA chain mRNA in panel (e) lane 3 relative to lanes 1 and 2 can be attributed to the increased total RNA loaded in lane 3 as is apparent in the 28S and 18S ribosomal bands.

Figure II-4.

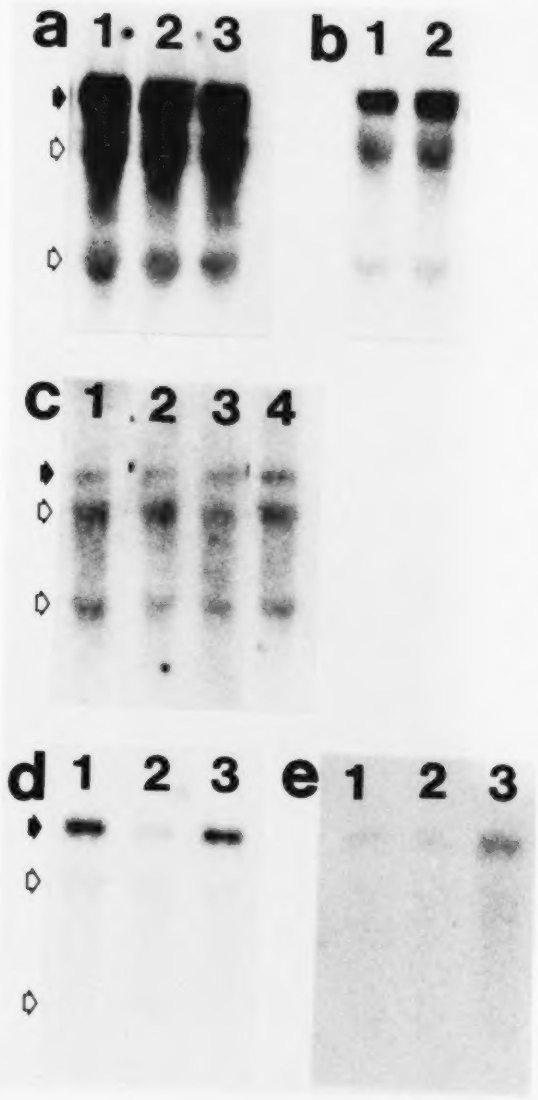
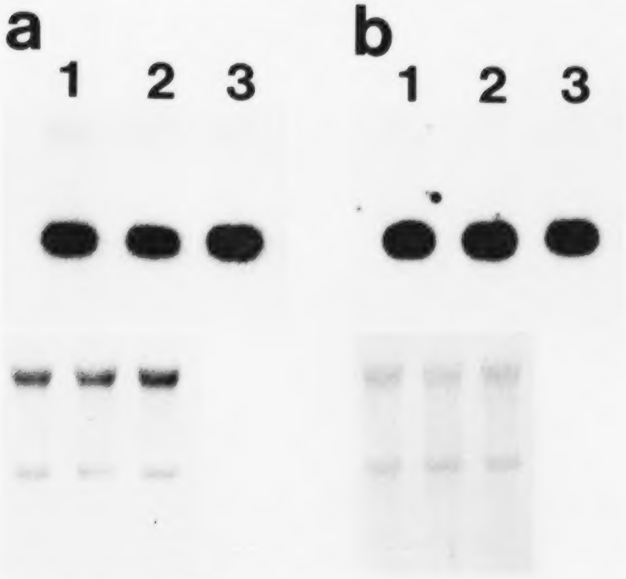


Figure 5. Northern blot analysis of oxytocin mRNA from the middle brain segment of (a) male and (b) female prepubescent rats. Upper and lower panels are as described in Figure 1. Lane 1 contains RNA from intact control rats at P20. Lanes 2 and 3 contain RNA from animals treated from P10 to P20 with estradiol (lanes 2) or testosterone (lanes 3).

Figure II-5.



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PART II

A NOVEL ROLE FOR OXYTOCIN IN HUMAN PARTURITION

CHAPTER III

LITERATURE REVIEW AND BACKGROUND INFORMATION

1. INTRODUCTION

In 1906 Dale demonstrated that the posterior pituitary contains a substance that has uterine stimulating properties. At the same time Blair Bell (1909) introduced pituitary extract into clinical obstetrics to induce labour and to stimulate post-partum uterine contractions. Crude extracts were initially used until the partially purified extract *pitocin* was produced by Kamm et al (1928). The efficiency of intravenous OT infusion was proposed by Page (1946) and introduced into clinical practice in 1948 by Theobald and colleagues. The subsequent development of purified synthetic OT (du Vigneaud et al, 1953) led to the widespread use of this hormone by slow intravenous administration to induce or augment labour. It was widely presumed that this treatment mimics the physiological release of OT which occurs as a normal component of the process of parturition.

An endocrine role for OT in the initiatian of human parturition remains controversial. This controversy is of considerable clinical importance in view of the fact that disorders of the timing of parturition remain by far the greatest cause of mortality and morbidity in the human reproductive process. The balance of evidence suggests that OT plays only a facilitatory role during the second stage of labour and after delivery but not in the actual initiation process itself. Although exogenous OT infusion will stimulate uterine contractions indistinguishable from normal spontaneous labour, maternal serum OT concentrations do not increase until labour has been well established (*vide infra*). In many species labour occurs normally in the absence of OT following hypophysectomy in the mother and without the presence of fetal OT as in fetal anencephaly (Little et al, 1956; Swaab and Oosterbann, 1983).

In the second part of this thesis I have reexamined the role and regulation of OT in parturition from a different perspective. We and others have proposed that human labour may be regulated by factors synthesized within the fetal membranes (amnion and chorion) and the maternal decidua. Amnion, chorion and decidua have substantial capacity to synthesize various peptides, steroids and prostanoids, adjacent to the likely target organ of these compounds, the myometrium (Siler-Khoder, 1988; Mitchell and Challis, 1988; Olson et al, 1983). Therefore I examined the novel hypothesis that OT is synthesized locally in these tissues and that changes in OT levels at the time of parturition thus could occur without affecting maternal circulating levels. These studies confirmed our hypothesis that the OT gene is expressed in human intrauterine tissues (amnion, chorion and decidua) and suggested that OT is regulated by sex steroids, possibly in a paracrine manner.

Numerous studies in the past two decades have provided evidence concerning the involvement of OT in the process of labour and these are discussed below.

2. PLASMA OT CONCENTRATIONS AND PARTURITION

2.1 DETERMINATION OF PLASMA OT BY BIOASSAYS

To obtain evidence for the role of OT in parturition, levels of circulating OT concentrations have been determined during pregnancy and labour in various animal species, as well as humans, initially by a variety of bioassay procedures. These assays measured either mammotonic or uterotonic activitiy of the hormone, using *in vivo* or *in vitro* models. The *in vivo* model quantitated intrauterine or intramammary pressure in lactating rats or guinea pigs following injection of extracted or unextracted plasma. The *in vitro* system used either mammary or uterine segments in organ bath systems. Following addition of plasma extracts, changes in tension were used as an index of activity. The results were compared to known standards. OT was usually extracted by procedures based on acid-alcohol, acetone-ether or gel filtration, to prevent degradation by oxytocinase, to remove interfering substances and to concentrate OT. Unfortunately, these bioassays lacked specificity and the variable results obtained by different investigators are summarized in table 1.

This variability may also reflect many other factors. For instance, the animal studies generally were performed with blood drawn from the external jugular vein that drains the pituitary. This may explain why the oxytocic activity in animal species is comparatively higher than that found in human studies, which generally measures OT concentrations in blood from the peripheral circulation. Coch et al (1965) compared the OT concentrations between blood drawn from the external jugular vein and an arm vein of women in labour. In advanced labour, and particularly during the second stage (after the cervix has been fully dilated), the oxytocic activity of the jugular plasma was 4-9 times greater than that of peripheral plasma, suggesting an increasing rate of OT secretion by the neurohypophysis during the expulsive phase.

Bioassays for OT (Table 1) suggested that oxytocic activity is low or undetectable during the first stage of labour and reaches a peak during the second stage. After parturition, there is a rapid decline in OT bioactivity to prepartum level within 10-30 minutes. However, bioassays lack sensitivity and specificity. The presence of oxytocinase, prostaglandins and other substances that influence smooth muscle contractility may compromise bioassay results by interfering with oxytocic responses. However, these observations suggested that OT is not necessary for the initiation of parturition, but that it augments uterine contractions at the time of expulsion of the fetus. Improvement in OT quantitation came with the development of radioimmunoassays (RIA) (Chard et al, 1970).

Table 1. Bioassay measurements of plasma OT concentrations (pg/ml)

Species	Extraction Procedure	Pre-labour	Stage of labour I	II	Post-partum	Reference
Sheep	Acetone	0-10	0-65	150-30000	0-10	Fitzpatrick, 1961
sheep	Acetone ether			560-3000		Fitzpatrick and Walmsley, 1965
Goat	Sephadex	0-20	6-90	90-300		Knaggs,1963
Goat	Sephadex			78-113		Chard et al
Rabbit				100-1200		Fuchs, 1966
Rabbit	Alcohol			6-500		Halder, 1970
Cows	unextr	0	0	0-3000		van Dongen and Hays, 1966
Cows	Sephadex	0-100	30-50	350-1000	0-400	Fitzpatrick and Walmsley1965
Horse	Sephadex	0-250	0-150	100-800	0-300	Fitzpatrick and Walmsely, 1965
Human	Acetone Ether			1390		Hawker et al, 1961
Human				140		Fitzpatrick, 1961
Human				300-900		Coch et al, 1965
Human	Sephadex			80-200		Fitzpatrick and Walmsley, 1965
Human			20-40	2000-4000		Coutinho et al,1964
Human			0	0		Vorherr 1972

2.2 DETERMINATION OF PLASMA OT BY RADIOIMMUNOASSAYS

2.2.1 Plasma OT concentrations during pregnancy

Using specific antibodies to OT, several investigators have measured maternal plasma OT concentrations during pregnancy and around the time of parturition (table 2). In animals, OT levels remain low throughout pregnancy except in rats, where OT levels have been reported to increase between days 18-21 (Kumaresan et al, 1973). However, the significance of this increase in OT levels is not known, as immunoneutralization of circulating OT impairs lactation but has no effect on the initiation of parturition in rats (Kumaresan et al, 1971; Higuchi et al, 1986). In goats and chronically catheterized sheep, maternal plasma OT levels do not increase until the expulsive phase of labour (Chard et al, 1970; Glatz et al, 1981), while in pregnant rhesus monkeys, plasma levels have been shown to increase with gestational age (Dawood et al, 1979). Similarly, a trend of gradually increasing but variable levels of OT and NP during late pregnancy is reported by several authors (Legros and Franchimont, 1972; Kumaresan et al, 1974; Vasicka et al, 1978; Dawood et al, 1979) and none by others (Chard et al, 1976; Leake et al, 1981; Sellers et al,1981). The disparity in the results among different studies may be due to differences in the specificity of antibody, extraction procedure and/or 'spurt' release of OT. OT, like other hypothalamic peptides, is released episodically and serial measurements have revealed fluctuating levels in the circulation. Thus the frequency and timing of blood sampling is important in determining the OT levels especially when the very short half-life of OT is taken into consideration. Most of these studies obtained one or very few samples from each patient and this may account for the wide variability observed in these studies. Recently, using a specific antibody to OT and frequent sampling, Fuchs et al (1991) have reported that basal levels of OT are undetectable throughout most of the pregnancy and do not increase near term.

Similarly, recent studies in sub-human primates have reported that basal plasma OT levels remain low during pregnancy, but a few weeks before parturition a circadian variation in maternal plasma OT occurs with highest levels at 2400 pg/ml. This nocturnal increase in OT concentrations correlates with nocturnal episodes of increased uterine activity that occur during the early hours of darkness (Honnebier et al, 1989; Seron-Ferre et al, 1989; Hirst et al, 1991). Furthermore, this uterine activity can be inhibited by administration of an OT antagonist (Honnebier et al, 1989). In many animal species, including primates, labour onset and parturition occur during night and early morning hours (King, 1956) suggesting that circadian variations in OT levels may facilitate or even initiate these processes. The source of this nocturnal increase in OT is not known. There is also an increase in uterine responsiveness to OT near term which causes myometrium to respond to small changes in OT concentrations (section 7). Whether a nocturnal increase in OT or uterine activity occurs in human remains to be examined. In general most investigators agree that during pregnancy maternal plasma OT levels remain low before labour onset.

Table 2. Radioimmunoassay measurements of plasma OT concentrations (pg/ml)

Species	Extraction Procedure	Pre-labour	Stage of labour		Post-partum	Reference
			I	II		
Goat	Fullers			78-113		Chard etal,1970
Goat	Fullers		1	9-115		McNeilly etal,1972
Sheep			5-20	5-250		Mitchell et al, 1982
Sheep		0.7	0.7	28	8	Glatz et al,1981
G pigs	Glass beads		0	50-1500		Burton et al,1974
Rabbit	Fullers	16	13.7	193	20	Fuchs and Dawood,1980
Rabbit		22		2486		O'Byrne et al, 1986
Cow	Acetone	0		30	0	Schams et al,1979
Horse	Fullers	0	11-15			Allen et al,1979
Rat		24-118				Kumaresan etal,1973
Rhesus		1-19				Seron-Ferre etal,1991
Rhesus			1-10			Honnebier et al,1989
Human	Glass bead	2-5		2-12		Chard et al, 1976,
Human	Fullers	1-35	20	61	32	Dawood et al,1978
Human	unextracted		72-164	64-199		Vasika et al, 1978
Human	unextracted		66-165	35-435		Kumaresan et al,1974

Human	Fullers	5-32			Dawood et al, 1978
Human		1.3	1.3	4.4	Leake et al,1981
Human		5	3-5	4.3	Sellers et al,1981
Human			3-23	1-47	Goodfellows et al,1983
Human		1.2	1.2	1.5	Fuchs et al,1991

2.2.2 Plasma OT concentrations during parturition

In order to understand the role of OT in the initiation of labour it is important to determine when the increase in maternal plasma OT concentration occurs. Human labour commonly is divided into stages. The first stage includes the time from labour onset until the cervix has been fully dilated. The second stage encompasses the time from full dilation to delivery of the fetus. This stage is accompanied by maternal bearing down efforts and is also known as the expulsive phase of labour. The third stage of labour lasts until the placenta is delivered. If an increase in plasma OT level occurs during stage 1 of labour then OT may be responsible for the initiation of labour. However, if the increase occurs during the expulsive stage of labour then OT may not be essential but facilitatory for the process of labour. There are few animal and human studies that measured maternal plasma OT both during pregnancy and stage 1 of labour. Although the maternal plasma OT levels were quantitatively different among most of the studies, no increase in plasma OT concentration occurred with onset of labour (table 2). Reported levels of plasma OT were higher in earlier radioimmunoassay studies compared to recent ones. These differences likely are due to methodology, nonspecificity of the antibodies or spurt release of OT (Chard et al,1970; Dawood et al,1978; Leake et al, 1981; Summerlee, 1981; O Byrne et al, 1986). The presence of estradiol-induced OT-like peptides that are measured by some antisera but not others, may also account for higher values reported in some studies (Amico et al, 1985; Amico and Hemple, 1990). Recently, Fuchs et al (1991) obtained blood samples at 1 minute intervals during stage 1 of labour and demonstrated that pulse frequency but not pulse amplitude increases during the stage 1 of labour compared to prepartum levels. These results have not been confirmed by others. Most investigators have concluded that the lack of change in plasma OT concentration during late pregnancy and early stages of labour in most human studies indicates that an increase in circulating OT levels is unlikely to be a requirement for the initiation of labour (Chard et al, 1970; Forsling, 1979; Leake et al, 1981; Sellers et al, 1981).

A consistent finding in most animal and human studies is that there is a rapid increase in circulating OT concentration during the expulsive phase of labour. OT levels peak at the expulsion of the fetus and declines during the third stage of labour. This increase in OT has been ascribed to Ferguson's reflex (Ferguson 1941). It is suggested that stretching of the birth canal acts as a stimulus (via the pelvic nerves) for the reflex release of OT from the neurohypophysis. The role of the Ferguson reflex in parturition is not clear. The OT surge during the expulsive stage of labour can be prevented by paracervical, spinal or epidural anesthesia, reinforcing the idea that the maternal OT surge at expulsion is a neurally mediated response to vaginal or cervical distension (Vasika et al, 1978). However, uterine contractions continue normally with these types of obstetrical analgesia. Conversely, spinal transection of pregnant rabbits had no effect on OT levels at the time of delivery. This suggests either that different factors or neural pathways may be involved in the stimulus for OT release during expulsion of the fetus or that the source of OT may be other than the neurohypophysis. In rats, bilateral section of the pelvic nerves slightly prolonged the duration of labour, but the blood levels of OT were not significantly different in pelvic neurectomized or sham operated controls (Louis et al, 1978; Burden et al, 1982, Higuchi et al, 1986). These data suggest that neural inputs leading to higher levels of OT at this stage are not necessary but may have a physiological role in improving the process of labour, enhancing expulsion of fetus and placenta and preventing uterine atony and postpartum haemorrhage.

3. METABOLISM OF OXYTOCIN

The levels of plasma OT during pregnancy and parturition also depend upon the metabolism of OT in the circulation, liver and kidney. During pregnancy, an aminopeptidase that can inactivate OT appears in the maternal circulation (Fekete, 1930). This enzyme was named *oxytocinase* because of its ability to hydrolyze the physiological substrate oxytocin. *Oxytocinase,* also known as cystine aminopeptidase, cleaves the pentapeptide ring of OT and VP between tyrosine and half-cystine residue with a free amino group. This action destroys the ring structure and all biologic activity of the molecule (Ryder 1966). The level of oxytocinase is low in non-pregnant women. It appears in the maternal circulation soon after conception and increases progressively with gestational age reaching a maximum at or near term and disappears rapidly after parturition (Tuppy, 1961; Babuna and Yenen, 1966). The placenta is usually thought to be the main source of this enzyme (Oya et al, 1974; Spellacy et al, 1977; Lampelo and Vanha-Perttula, 1980) but it is also present in other tissues including, liver, kidney, pancreas and ovary (Roberts, 1977). The exact role of oxytocinase in pregnancy is not known. Various studies assessing its physiological significance have provided conflicting results as to whether it is involved in the maintenance or termination of pregnancy (Roy and Karim, 1983; Roy et al, 1985). Gazerek et al (1976) have noted that activity of this enzyme increases until the 38th week of gestation and declines thereafter. While others found that a significant fall in serum oxytocinase occurs during the active phase of labour as compared to the levels before labour (Ances, 1972; Kilmek and Bieniarz, 1969; Wieczorek and Sobiech, 1980). This suggests a decrease in the metabolism of OT before or during parturition thereby increasing the concentration of circulating OT when it is most needed. To the contrary, several authors did not find any decrease in plasma oxytocinase activity during late pregnancy or parturition (Roy and Karim, 1983; Babuna, 1961; Floyd et al, 1973; Christensen and Hagelid, 1974; Edeberi et al, 1989). A high level of enzyme activity is also detected in amniotic fluid during early pregnancy and this decreases as pregnancy advances (Roy et al, 1986). The decreasing oxytocinase activity in the amniotic fluid may be responsible for increasing levels of oxytocin in amniotic fluid at term. However, the significance of amniotic fluid OT in the initiation of labour is not known. High oxytocinase activity is also found in the uterus, and this may have physiological significance during pregnancy or labor (Roy et al, 1985). Prostaglandins have been shown to inhibit oxytocinase activity in human pregnancy serum and placenta (Roy et al, 1981; 82;).

Consistent with increasing oxytocinase activity, there is also a significant increase in the metabolic cleareance rate (MCR) of OT during pregnancy (Thornton et al, 1990). In contrast, other authors have reported that the MCR of OT was unaffected by pregnancy and was similar to that found in males (Dawood et al, 1980) and nonpregnant females (Fabian et al,1969). This may be due to a difference in methodology or failure to completely inhibit oxytocinase activity following sample collection leading to spurious results.

It is not certain whether OT is the prime target for oxytocinase or whether its principle substrates are other peptides.

Other enzymes similar to, but not identical to oxytocinase are also present in the serum and placenta and may play a role in degradation of OT (Lampelo and Vanha Perttula, 1980; Lalu et al, 1984; 1986; Watanabe et al, 1989).

4. ROLE OF THE MATERNAL PITUITARY IN PARTURITION

The source of OT during pregnancy and parturition is considered to be the hypothalamus and posterior pituitary. Direct and indirect evidence implicating the role of neural OT in parturition comes from the rat, guinae pig and rabbit. Electrical stimulation of the hypothalamus or posterior pituitary in rabbits induces uterine contractions and delivery (Fuchs and Saito, 1971) while hypophysectomy or lesions placed higher in the neuro-hypothalamo-hypophyseal system caused prolonged pregnancy or protracted labour (Chez et al, 1970). There is also an increase in biosynthesis of OT in the supraoptic and paraventricular hypothalamic nuclei in term pregnant rats and immediately following parturition (Caldwell et al, 1987; Zingg et al, 1989) and this has been suggested to play an important role in maternal behaviour and lactation. In contrast, in several animal species including the human (Little et al, 1958), maternal hypophysectomy during pregnancy had no effect on the initiation of labour but impaired milk secretion and milk ejection (Averill and Purves, 1963; Gale and McCann, 1961). With very few studies it is difficult to draw any conclsions but the general consensus is that OT release from the maternal pituitary is not necessary for the initiation of labour or birth of the fetus(es).

5. ROLE OF THE FETAL PITUITARY IN PARTURITION

In human, the association of fetal anencephaly, in which the fetal pituitary is atrophic, and prolonged pregnancy is widely used as evidence that the fetal pituitary may play an important role in spontaneous labour (Malpas 1933). Chard et al (1971) found higher OT levels in human umbilical cord arterial plasma than in cord venous plasma, while no OT was found in the maternal plasma during the second stage of labour. This and the lack of OT in cord plasma of an anencephalic fetus suggested that the fetal pituitary might be involved in the release of OT during labour. This was followed by determination of OT levels in fetuses and newborns by several investigators (table 3). Normal human and rhesus monkey fetuses at delivery have significantly higher cord blood levels of OT compared to levels measured in maternal peripheral blood. Furthermore, the arteriovenous difference in the cord blood levels is higher following spontaneous labour onset than in the elective cesarean section group (Dawood et al 1978; Sellers et al, 1981). These data suggest that elevated OT during labour may be of fetal rather than maternal origin and, therefore, that the fetal brain may influence the timing of labour onset.

There are two major considerations when evaluating the possible role of fetal OT in the initiation of labour. The first is whether OT is synthesizeed by the fetus and the second is whether it can be transported to the maternal myometrium to effect contractility.

OT concentrations in fetal plasma have been measured in several animal species. During early pregnancy, OT concentrations are low or undetectable in the sheep and pig fetus (Alexander et al, 1974; Forsling et al, 1975; McDonald et al, 1979). During late pregnancy in guinea pigs, sheep and baboons, OT concentrations in the fetal posterior pituitary or fetal plasma are higher than the maternal levels (Burton and Forsling, 1972, Forsling et al, 1975, Mitchell et al, 1982, Dawood et al, 1979). However, the significance of fetal OT in the mechanism initiating parturition is not understood. In the rat the processing of fetal OT is not completed until after birth and it is not known whether the precursor forms of OT have any biological activity. Recently an OT-like material in the plasma of neonates has been reported, the significance of which is not known (Gore-Ervin et al, 1988). It could be one of the precursor form of OT. Processing of OT in the human fetal pituitary has not been studied.

The second consideration concerns transplacental passage of OT from the fetal to maternal circulation and the possibility of degradation by placental oxytocinase. In spite of the presence of oxytocinase in the placenta, both direct and indirect evidence from very few studies suggest that transplacental passage of OT occur in both directions significance of which is not cleary understood. In the guinea pig and baboon, administration of OT to the fetal circulation results in a rise in OT concentrations in the maternal circulation within a few minutes (Burton et al, 1974; Dawood et al, 1979). In the sheep, OT crossed the placenta and induced uterine contractions when injected into the fetal circulation near term. These studies seemed to indicate that OT is not significantly degraded in the fetal circulation or during placental transfer and is capable of stimulating uterine contractions (Nathanielsz et al, 1973). However, there were no controls and the number of animals were small in these studies rendring the interpretation difficult. Indeed, recent studies by the same group have demonstrated spontaneous uterine contractions at this stage of gestation in the absence of fetal OT infusions. In the rhesus monkey, no increase in uterine activity was observed following injection of OT into the fetal circulation (Hirst et al, 1991). This could be due to a species difference. With the small number of studies, it is difficult to interpret the effect of fetal OT on uterine contractility and initiation of labour.

The stimulus for the release of OT in the fetus during and after labour is not known. From these data, it is not clear whether it is the cause or an effect of labour. In women Vasicka et al (1978) found higher OT levels in umbilical blood than maternal venous blood if the infants were born with signs of asphyxia. However, hypoxemia and haemorrhage had no effect on the levels of OT in the sheep fetus (Forsling et al 1979, Stenger et al, 1984).

Evidence against the role of fetal OT during parturition include studies on women who carried anencephalic fetuses without hydramnios. The mean gestational length in this group of patients was similar to a normal control group suggesting that the timing of labour onset in human is not determined by fetal OT. Furthermore, an increase in plasma OT concentration at the time of delivery in sheep fetuses (Forsling et al, 1975) was not confirmed by Glatz and Mitchell (1980), in spite of frequent sampling. Decapitation of rabbit fetuses or aspiration of rat fetal brain had no effect on gestational length though labour itself was protracted (Jost 1973, Swaab and Honnebier, 1973). Finally, the removal of the fetal baboon at midtrimester while leaving the placenta intact does not initiate labor. The uterus continues to grow and labour occurs at term (Albrecht et al, 1989). In contrast, following fetectomy in pregnent rhesus monkeys, the placenta was retained for a longer period of time (more than 2 weeks past normal term). Though it is difficult to rationalize these apparent interspecies differences, it appears that fetal OT may facilitate the course of labour but is unlikely to be involved in the initiation process.

The possible explanation for the presence of OT in the fetal circulation may be that maternal OT can cross the placenta. This is supported by the fact that the umbilical artery-vein gradient can be effectively reversed when OT is administered to the mother during labour (Noddle, 1964; Kato, 1974; Dawood et al, 1979). Furthermore, detection of OT in the umbilical artery of liveborn anencephalic infants and the increasing level of OT in fetal blood with successive deliveries in guinea pigs suggests that OT in the fetal circulation may be of maternal origin (Oosterbaan et al, 1983; Burton et al, 1974). But why umbilical artery-vein gradient in OT is difficult to explain on this basis. Pulsatility of OT secretion and slow degradation of OT in the fetal circulation may partially explain this difference. A source of OT other than the fetal pituitary also could explain the presence of OT in the absence of a fetal pituitary.

6. OT IN AMNIOTIC FLUID

The concentration of OT in the amniotic fluid of pregnant women increases significantly from 8 pg/ml in the first trimester to 44 pg/ml at term. It has been suggested that the main source of amniotic fluid OT is fetal urine or direct efflux from the cord vessels (Seppala et al, 1972, Dawood et al, 1978). In rats, maternal OT does not appear to contribute substantially to the amniotic fluid OT (Oosterbaan et al, 1985).

Measurements of OT taken around the time of labour onset have given conflicting results. Some investigators found that OT levels were higher in the amniotic fluid samples from women in labour compared to those collected prior to labour (Seppala et al, 1972, Dawood et al, 1977; Dawood et al, 1979). Conversely, no increase in amniotic fluid OT during spontaneous labour and similar levels of

Table 3. Comparative concentrations of OT in maternal and fetal plasma and in amniotic fluid (pg/ml).

Author	Chard	Dawood	Seppala	Oo'baan	Sellers	Fuchs
Umbilical Artery						
SL*	54	116		40	90	101
CS**	24	29		34	50	
Umbilical Vein						
SL	30	38		17	40	55
CS	16	9	20			
Maternal Plasma						
SL	4-36	64	9	65	41	
CS			12			
Am.Fluid						
weeks	8					
weeks	44					
Weeks	30					
SL				15		
CS		11				

* samples collected following spontaneous labour

** samples collected at elective cesarean section at term prior to labour amniotic fluid OT in normal and anencephalic fetuses indicate that sources other than the fetal pituitary contribute to OT in the amniotic fluid (Oosterbaan and Swaab, 1989). Immunoreactive OT has been detected in the fetal zone of the adrenal cortex (Ravid et al, 1986). So far there are no data to suggest that amniotic fluid OT plays any role in parturition except one indirect study where a higher concentration of OT was detected in meconium stained amniotic fluid and the duration of the second stage of labour was shorter in those women (Seppala et al, 1972). Although it may be possible that OT from the amniotic fluid can diffuse through the membranes and stimulate the myometrium, there is no evidence that this occurs.

In conclusion, the above studies suggest that neither maternal nor fetal OT plays a major role in the initiation of labour in the human. Based on measurements of OT in the maternal and fetal circulations, OT may have a facilitatory role during parturition but the source of this OT is not known. There is evidence both in favour and against the maternal and fetal pituitary as sources of OT during pregnancy.

7. OT RECEPTORS IN UTERINE AND INTRAUTERINE TISSUES

The nonpregnant human uterus contains high affinity binding sites for OT with an apparent Kd of 1-3 nM (Fuchs et al, 1982, 84; 1990). The relative concentration of OT receptors in the nonpregnent uterus is much lower than that of the pregnant uterus

(Fuchs et al, 1984b). The concentration of OT receptors in the myometrium increases throughout pregnancy. In the early second trimester OT receptor concentrations are six-fold, and in late pregnancy about 80-fold, higher than nonpregnant levels (Fuchs et al, 1984). A further 2-3 fold increase in receptor concentration occurs around the time of parturition. This change in receptor concentration parallels the increased uterine sensitivity to OT at each of these times. Maximal myometrial sensitivity to OT occurs during labour (Caldeyro-Barcia and Serona, 1961). Concerning the role of OT in initiation of labour, several authors have suggested that labour commences not because of increased secretion of OT but when the myometrium becomes sufficiently sensitive to circulating levels of OT (Theobald, 1959; Takahashi et al, 1980; Fuchs et al, 1983). Similarly in rats, OT receptors increase significantly about a day before labour onset and exogenous OT will induce parturition only when administered less than 24 hours before labour (Soloff et al, 1980; Fuchs and Poblete, 1970). The increasing sensitivity of the myometrium to basal levels may preclude the need for an increase in plasma levels of OT to initiate labour. Though little is known about the regulation of human myometrial OT receptors, in both sheep and rats estrogen increases OT receptor synthesis whereas progesterone prevents the estradiol-induced increase (Soloff, 1975, 1988; Nissenson, 1978).

OT binding sites also are present in the human endometrium and in decidua, amnion and chorion at term (Fuchs et al, 1982; Bennedetto et al, 1990). The binding affinities of OT receptors in fetal membranes and decidua are similar to myometrial OT receptors and do not change with parturition. The density of decidual OT receptors increases in parallel to myometrial OT receptors during pregnancy and parturition.

In addition to its direct action on uterine contractility, OT stimulates prostaglandin (PG) synthesis in fetal amnion, chorion and decidua. The capacity of intrauterine and uterine tissues to produce prostaglandins increases with estradiol and may be of some importance in labour.

Recent cloning of the human OT receptor revealed that it is a member of the 7-transmembrane domain receptor protein family. The 4100 base pair OT receptor cDNA encodes for a 388 amino acid protein with a relative molecular mass of 42 kDa (Kimura et al, 1992). Two sizes of OT receptor mRNA are present, 3.6 kb in mammary gland and 4.4 kb in uterine, endometrial and ovarian tissues suggesting tissue specific regulation of OT mRNA. Expression of the OT receptor gene increases significantly in association with parturition.

8. MECHANISM OF ACTION OF OT

Binding of OT to its receptor stimulates the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (Rivera et al, 1990). IP_3 stimulates uterine contractility by increasing the release of intracellular calcium from the sarcoplasmic reticulum in myometrial cells (Cartsen et al, 1985; Flint et al, 1986; 1986; Schrey et al, 1986). Free intracellular calcium binds to the Ca^{++} binding protein calmodulin. This complex binds to and activates myosin light chain kinase, which catalyzes the phosphorylation of the light chain subunit of myosin and results in contraction (Aksoy et al, 1983). DAG stimulates protein kinase C, which catalyzes the phosphorylation of proteins involved in a number of other cellular processes (Michell, 1982; Angel et al, 1988). One of these processes includes the stimulation of prostaglandin synthesis in amnion and decidua.

In amnion, DAG release following hydrolysis of PIP_2 stimulates protein kinase C, which leads to an increase in the rate-limiting enzyme in PG synthesis, prostaglandin H_2 synthase (PGHS). In addition, DAG lipase and monoacylglycerol lipase act on DAG to release arachidonic acid, the obligate substrate for PG synthesis (Okazaki et al, 1981). Thus, OT increases the enzyme activity as well as substrate supply for PG synthesis. Further evidence consistent with the interaction between OT and PG includes an increase in circulating PG metabolites following induction of labour with OT (Husselein et al, 1981, 1983).

Paradoxically, PG itself causes further stimulation of OT release when administered to pregnant women, rats or nonpregnant sheep (Chan, 1977; 1980; 1992; Gillespie et al, 1972; Flint et al, 1982; Roberts et al, 1976). The mechanisms involved in PG stimulation of OT release are not known. Thus both OT and PG stimulate each others' release, resulting in a synergistic effect on the myometrium.

The OT receptor is coupled to calcium magnesium (Ca^{++} Mg^{++}) ATPase. OT and its analogue inhibit Ca^{++} Mg^{++} ATPase and the level of inhibition corresponds to the potency and binding affinity of the OT analogues. Ca^{++} Mg^{++} ATPase is coupled to the calcium pump and inhibits extrusion of calcium resulting in the prolongation of calcium interaction with the contractile proteins. The importance of inhibition of calcium extrusion relative to stimulation of uptake of extracellular calcium or release of calcium from intracellular stores is not known (Soloff et al, 1972; Akerman et al, 1979).

9. PERIPHERAL SYNTHESIS OF OT

OT, although primarily regarded as a neurohypophyseal hormone, also has been identified within several peripheral tissues including the gonads, adrenal medulla, placenta, pancreas and thymus (Noteborn et al 1988, Amico and Finn 1988, Dave et al 1988, Lefebvre et al, 1992).

OT is synthesised in the corpora lutea of many species including cow (Fields et al, 1983; Wathes et al, 1983; Flint et al, 1990), pig (Einspamer et al, 1986), cynomolgus monkey (Khan Dawood et al, 1984) and human (Dawood and Khan-Dawood, 1986; 1991). It is synthesized by the granulosa-derived large luteal cells and periovulatory granulosa cells (Eispamer et al, 1986; Fehr et al, 1987; Jung Clas and Luck, 1986; Plevrakis et al, 1990;). Post translational maturation of pro-OT/neurophysin in the ovaries proceeds by the same proteolytic events as those in the neurohypophyseal system. The significance of ovarian OT synthesis is not known but it may play a role in ovarian steroidogenesis, ovulation and demise of the corpus luteum (Grazul et al, 1989; Jarry et al, 1990; Pitzel et al, 1988, 1990; Wathes et al, 1992).

11. ESTROGEN AND PROGESTERONE IN PARTURITION

11.1 ESTROGEN

In most species, estrogen and progesterone are important in the mechanism of parturition (Thorburn and Challis, 1979). Regulation of myometrial contraction is believed to be mainly hormonal. Estrogens have several actions on the myometrium that are consistent with a role in preparation of the uterus for parturition including increasing the synthesis of the contractile proteins actin and myosin that are necessary for contractile activity (Clark ad Peck, 1979). Furthermore, estrogens are involved in the synchronization of myometrial contraction by elevating the formation of gap junctions that permit electrical coupling of contiguous muscle cells. This action includes the induction of connexin mRNA and protein synthesis by estrogen

(Verhoff and Garfield, 1986; Lye et al, 1991). Estrogens increase the sensitivity of the myometrium to uterotonics by increasing the expression of myometrial receptors for OT (Soloff 1975, Nissenson et al, 1978) and α-adrenergic agonists (Roberts et al 1981). In contrast, progesterone increases receptors for inhibitory β-adrenergic agonists and decreases OT receptor concentrations. Another action of estradiol at parturition may be to affect PG endoperoxide metabolism. In cultured decidual cells estrogen increases PGE_2 synthesis, an effect that is likely mediated by an estrogen-induced increase in PG endoperoxide synthesis (Olson et al, 1983). In addition to a direct effect on PGHS, estrogen may stimulate phospholipase A_2 (PLA_2) levels in intrauterine tissues. An increase in PLA_2 would lead to liberation of arachidonic acid and a further increase in PG production.

In pregnant women, there is a linear increase in the concentration of estrogens in the maternal circulation throughout gestation. At term, the concentrations of estradiol, estrone, estriol and their corresponding conjugates are more than 10-fold greater than normal levels in ovulatory women. Most of the estrogens originate from the placenta, which is dependent upon the maternal and fetal adrenals for the supply of androgen precursors. The weight of the human fetal adrenal glands increases progressively after 20 weeks of gestation and the growth is particularly great in the last 4-6 weeks of gestation. This increase in size is largely due to enlargement of the fetal zone of the adrenal cortex, which is the source of secreted dehydroepiandrosterone sulfate and other androgenic estrogen precursors (Carr and Simpson, 1981). Steroid secretion by the fetal adrenals at term may be more than 10-fold that of the adult adrenals (McDonald and Porter, 1977, Siiteri and McDonald 1966).

11.2 PROGESTERONE

Progesterone synthesis increases throughout pregnancy (Dorr et al, 1989). Low density lipoprotein-cholesterol serves as the major precursor for placental progesterone synthesis. In many species, progesterone has a pivotal role in maintenance of pregnancy and progesterone withdrawal with a coincident increase in plasma estradiol concentration leading to the initiation of parturition (Thorburn and Challis 1979; Challis and Olson, 1988). This mechanism has been well elucidated in the sheep model. In this species, an increased fetal cortisol output by the sheep fetus near term induces 17α-hydroxylase and 17,20-lyase in the placenta, thereby promoting the conversion of progesterone to estrogen and increasing the estrogen/progesterone ratio. At term, this increase in the estrogen/progesterone ratio above a critical threshold prepares the myometrium for synchronized contractions in response to a variety of stimuli.

The refractoriness of the myometrium to OT and PGs, under progesterone dominance, may be due to inhibition by progesterone of estradiol receptors or by the induction of synthesis of certain inhibitory proteins (Clark et al, 1977). The threshold dose of OT required to stimulate myometrial activity decreases by 90% following DES administration to sheep at day 130 of gestation (Liggins et al, 1972). Similarly, in rabbit maternal plasma, progesterone decreases at the time of parturition and uterine excitability increases. This response may be blocked by exogenous progesterone.

There is little evidence to support an inhibitory role for systemic progesterone in the control of uterine activity in either humans or non-human primates. In rhesus monkeys circulating progesterone remains elevated until delivery of the placenta thus demonstrating that labour and delivery may occur in primates without a decline in plasma levels (Turnbull et al, 1974). Therefore various investigators have examined the possibility that changes in steroid production may occur via a paracrine system within the intrauterine tissues (amnion, chorion and decidua) themselves and thus may not be reflected in maternal plasma.

11. HUMAN FETAL MEMBRANES (AMNION, CHORION) AND DECIDUA

11.1 AMNION

Amnion is the innermost of the two fetal membranes and is in contact with amniotic fluid inside and chorion on its outer surface. Amnion is derived from the cytotrophoblastic cells of the outer cell mass immediately adjacent to the dorsal aspect of the germ disc, at about the seventh day of development of the normal fertilized ovum (Hertig, 1945). The amnion forms a fluid filled, membranous sac that surrounds the embryo and later the fetus. With distension of the amniotic sac, the amnion comes to lie in apposition with chorion. Amnion and chorion are adherent but easily separated from each other.

The amnion covering the inner surface of the placenta is called placental amnion, while the rest of the amnion is termed reflected amnion. The amnion is a complex multilayered structure that has been well described by Bourne (1966, 1973). He has described five layers in the amnion, including the epithelium, a basement layer, a compact layer, a fibroblastic layer and a spongy layer. The innermost layer, the epithelium, faces the amniotic cavity and consists of a single layer of nonciliated cuboidal cells. The epithelium lies on the basememt membrane next to which lies an acellular compact layer which is resistant to leucocytic infiltration. The underlying fibroblast layer consists of fibroblasts and Hofbauer cells (macrophages) in a mesh of reticulin. It forms a considerable part of the thickness of amnion. The spongy layer, which is an intermediate layer between chorion and amnion, is made of a reticular network of many collagen like bundles. It is capable of significant distention and the mucus contained within its structure allows it to move upon the underlying fixed chorion.

11.2 CHORION

The chorionic membrane lies adjacent to the outer surface of the amniotic membrane and separates amnion from decidua. During early pregnancy, trophoblastic cells proliferate and cover the entire conceptus. The chorionic villi in contact with the decidua basalis proliferate to form the leafy chorion which will become the placenta. By the fourth month, when the developing gestational sac obliterates the uterine cavity, the peripheral chorionic villi in association with the decidua capsularis cease to grow and become compressed. These villi subsequently degenerate producing the smooth avascular membrane known as the *chorion laeve*.

The chorion laeve consists of four layers, including a cellular layer, a reticular layer, a pseudobasement membrane, and a layer of reticular and trophoblastic cells. The cellular layer is best seen early in pregnancy and often is absent at term. The reticular layer is made up of a network of reticulin in which fibroblasts and Hofbauer cells are embedded. Reticular fibers from this layer penetrate deeply into the trophoblast to bind it to the underlying uterus. The trophoblastic layer varies in depth and at term it consists of from two to ten layers of trophoblast cells. The outer surface of trophoblast layer is not well distinguished from the decidua capsularis to which it becomes attached. The trophoblast layer also contain ghost or residual villi, the remains of chorionic villi.

11.3 DECIDUA

Decidua is the stromal part of the endometrium transformed by the hormonal milieu of early pregnancy. Under the influence of progesterone, endometrial stromal cells enlarge to form rounded decidual cells. The portion of decidua directly beneath the site of implantation is termed the *decidua basalis* and forms the maternal component of placenta. The *decidua capsularis* surrounds the developing fetus and remainder of the uterine cavity is lined by *decidua vera* or *decidua parietalis*. By the fourth month, with continuous growth of the gestational sac, the decidua capsularis fuses with the decidua vera. Perhaps due to the stretching caused by this rapid growth, the decidua vera decreases in thickness from about 1 cm in the first trimester to 1-2 mm at term. Decidua vera is composed of three layers, a surface or compact zone (*zona compacta*), a middle portion or spongy zone (*zona spongiosa*) with glands and numerous blood vessels and a basal zone (*zona basalis*). At the time of delivery, the decidua separates from the uterine cavity at the zona spongiosa. The basal zone remains after delivery and gives rise to new endometrium. The compact zone consists of lightly staining, large, closely packed epithelial cells with round vesicular nuclei. Numerous small round cells with very scant cytoplasm are scattered among typical decidual cells. In early pregnancy, the spongy layer consists of large distended glands separated by minimal stroma. At term these glands have a cuboidal or flattened epithelium and degenerate.

An interesting feature of decidua is that, at term, approximately 50% of cells are of bone marrow origin (Bulmer et al, 1988; Vince et al, 1990). These cells, usually associated with the immune system, include tissue macrophages, large granular lymphocytes, T cells and other leucocytes. This supports speculation regarding a possible role for the immune system in the mechanism of parturition.

11.4. STEROID SYNTHESIS IN AMNION, CHORION AND DECIDUA

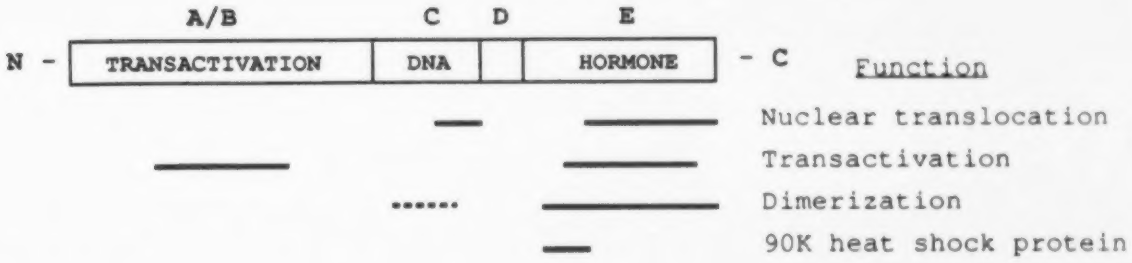
The amnion, chorion and decidua are metabolically active and have a substantial capacity for steroid production (Mitchell et al, 1982; Mitchell and Powell, 1984; Gibb and Lavoie, 1980, 1981; Romano et al, 1987; Mitchell et al, 1988). The most important precursors for steroidogenesis in these tissues are the steroid sulfoconjugates which are cleaved by sulfohydrolase enzymes to form free steroids. Estrone sulfate and dehydroepiandrosterone sulfate serve as precursors for estrone and estradiol. Progesterone is formed from pregnenolone sulfate. In the rhesus monkey, amniotic fluid estrone and estrone sulfate concentrations rise in late pregnancy (Mitchell et al, 1980; Turnbull et al, 1977). The sulfohydrolase enzymes which convert sulfurylated steroids to free steroids are present in chorion, decidua, amnion and placenta (Gant et al, 1977). The activity of these sulfohydrolase enzymes may be an important regulator of the free, biologically active concentrations of estrogen and progesterone within these tissues.

We and others have examined the possibility that changes in steroid production may occur within the intrauterine tissues and affect peptide or prostanoid synthesis in a paracrine or autocrine manner. There is a significant increase in the ability of fetal membranes to hydrolyze estrone sulfate at term. Estrone may be converted to the more potent estradiol by the enzyme 17β,20α-hydroxysteroid dehydrogenase. This same enzyme also reduces progesterone to its inactive metabolite 20α-DHP. This enzyme is present within amnion and chorion tissue (Mitchell and Challis, 1988). A change in this enzyme locally thus could increase the activity of the estrogens present and, at the same time, decrease the activity of progesterone. The combination of these changes may cause a significant increase in the local estrogen/progesterone ratio within intrauterine tissues, without significantly altering peripheral plasma estrogen or progesterone concentrations at term. The amnion, chorion and decidua are situated in juxtaposition to the myometrium. Any changes occurring in these tissues at the time of parturition may influence myometrium in a paracrine manner. These changes may lead to other effects that may prepare the myometrium for the trigger to the onset of labour.

12. SEX STEROID RECEPTORS IN FETAL MEMBRANES

Estrogen and progesterone belong to the steroid receptor superfamily of ligand-dependent transcription regulatory proteins that also includes nuclear receptors for thyroid hormone, glucocorticoid hormones, retinoic acid and vitamin D3. The primary function of the nuclear receptor is to regulate the rate of transcription of certain genes by binding as a hormone receptor complex to specific sequences termed *hormone response elements.* These interactions may either stimulate promoter activity, by acting as enhancers, or repress transcription, probably by interfering with the activity of other promoter elements. Evidence obtained from cDNA cloning, sequence alignment, deletion studies, and domain swap experiments, indicates that these receptor proteins consist of three discrete functional domains (Fig III-2.), a variable amino-terminal transactivation domain, a well-conserved DNA binding domain (DBD) and a carboxy-terminal steroid binding domain (SBD) (Krust et al 1987; Beato et al, 1989; Ham and Parker, 1989).

Figure III-1. Schematic diagram of the general stucture and functional organization of steroid hormone nuclear receptors.



12.1 ESTROGEN RECEPTORS

The estrogen receptor (ER) protein consists of 595 amino acids and is 66 kDa in size. The mRNA is reported to be 6.2 kb in size. Before hormone activation and under hypotonic conditions, steroid receptors form large, oligomeric inactive complexes when isolated from target cells. This inactive state is maintained by association of ER protein with cellular heat shock proteins (hsp 90) in a complex which is unable to bind DNA. Binding of the estrogen to the *hormone binding domain* causes the receptor to dissociate from hsp 90, dimerize with other active forms of ER and translocate to the nucleus for DNA interaction. A transactivation sequence is also located in the HBD and is dependent upon the binding of a functional ligand. Binding of effective anti-hormones can promote nuclear localization, but will prevent transactivation. The ER closely resembles the thyroid hormone and retinoic acid receptor (Beato, 1989; Ham and Parker, 1989).

12.2 PROGESTERONE RECEPTOR

Similar to ER, the progesterone receptor (PR) is structurally organized into three major functional domains. The PR resembles the glucocorticoid, mineralocorticoid, and androgen receptor. In human breast cancer cell lines, PR mRNA was detected in 4 main bands at 5.1, 4.3, 3.7 and 2.9 kb by Northern blot analyses

(Misrahi et al, 1987). The PR is unique among steroid receptors in that it is expressed as two different sized proteins termed PR-B (120 kDa) and PR-A (94 kDa). PR-A is a truncated form of PR-B that is missing 165 N-terminal amino acids that are present in PR-B. The A and B forms of PR may be produced from the same gene either by alternate initiation of translation at two different AUG start sites within the same RNA transcript, as observed in chicken PR. Alternatively, two promoters may give rise to two distinct PR mRNAs as occurs in human breast cancer cell lines (Conneely et al, 1987; Christensen et al, 1991). The transcription of a single gene from multiple promoters provides additional flexibility in the control of gene expression. It is possible that both promoters can be expressed in different cell types or they may be differentially regulated in the same cell type. Although both transcripts are inducible by estradiol, only half-palindromic ERE was found in PR-A and no candidate for an ERE could be detected in the promoter sequence for PR-B. It is not known whether the half-ERE of PR-A can mediate the estradiol effect (Kastner et al, 1990). PR-A and PR-B have different target gene specificities. An inhibitory role for the N-terminal domain of PR-B, independent of DNA binding specificity, has been suggested. In addition, this region may help to determine gene specificity as several lines of evidence suggest that the N-terminal region of the steroid receptor is involved in modulating transcriptional responses of specific target genes (Beato et al, 1989).

12.3 ER AND PR IN AMNION, CHORION AND DECIDUA

The status of ER and PR within intrauterine tissues was determined by several investigators (Khan Dawood and Dawood, 1984; Padayachi et al, 1989a, b, Haluska et al, 1990). Binding as well as immunocytochemical studies showed that both ER and PR are present in decidual tissue and decrease with advancing gestation. This may be due to the inhibitory effect of higher levels of steroids since treatment with the progesterone antagonist RU-486 increases ER in the decidua of the rhesus monkey (Haluska et al, 1990). The physiological significance of this finding is not known but, at least in the rhesus monkey, there appear to be no changes in ER and PR concentrations occurring around the time of spontaneous labor onset. Using binding studies with amnion and chorion, there was no detectable ER or PR at any stage of gestation or following parturition (Khan-Dawood and Dawood, 1984; Brenner et al, 1991). This could mean that these tissues are devoid of ER and PR. It also may be due to a low sensitivity of the assay or to methodological problems secondary to the presence of high tissue concentrations of estrogen and/or progesterone. The high steroid levels may either interfere with the assay or the highly steroid-dominated environment may inhibit induction of both estrogen or progesterone receptors.

In general, the major regulators of steroid receptor concentrations are the ligands themselves (Clark et al, 1977; Okulicz et al, 1981; Takeda et al, 1986). Although the precise mechanism of this autoregulation is not known, many studies have investigated the complex interactions between the sex steroids and their receptors (Isomas et al, 1989; Saceda et al, 1988; Rosewicz et al, 1988). More studies with sensitive techniques are required to determine the ER and PR status of fetal membranes and decidua in order to understand their effect in these tissues.

12.4 HORMONE RESPONSE ELEMENTS

Activated estradiol and progesterone receptors mediate their effects by binding to their corresponding steroid response elements in the DNA. This is briefly discussed here to introduce a discussion of the regulation of OT gene expression by steroids. ERE contains an inverted repeat of the sequence TGACC (AGGTCAnnnTGACCT). This sequence can be converted into a GRE/PRE by changing one or two bases (Fig III-2.)(Martinez et al 1987). This suggests that steroid response elements constitute a family of related sequences (Beato 1989). EREs and thyroid response elements (TRE) have identical half-sites but their spacing is different. ERE half-sites are separated by three nucleotides whereas the TRE lacks the three central non-conserved nucleotides between the half-sites (Martinez et al 1987). The thyroid hormone receptor can bind to an ERE but is transcriptionally inactive (Glass et al, 1988) and can therefore inhibit estrogen induction of transcription by competing for the ER target sequence.

Figure III-2. Concensus hormone responsive elements for the steroid receptors.

gluccocorticoid
progesterone
androgen
mineralocorticoid
} responsive element **G G T A C A n n n T G T T C T**

estrogen responsive element **A G G T C A n n n T G A C C T**

thyroxin
retinoic acid
} responsive element **T C A G G T C A——T G A C C T G A**

The progesterone response element (PRE), defined by gene transfer and footprinting experiments, contains a conserved hexanucleotide sequence, TGTTCT. This forms part of an imperfect inverted repeat of 15 bp in which the three central nucleotides are not conserved. Interestingly TGTTCT inverted repeats can also act as glucocorticoid and androgen response elements (Strahle et al 1987, Ham et al 1988). Although the same GRE/PREs mediate glucocorticoid, progesterone, mineralocorticoid or androgen responses, it has been proposed that the base contact points of glucocorticoid and progesterone receptors may not be identical.

These regulatory elements do not exist as isolated pieces of DNA *in vivo* but rather are arranged in complex chromatin structures. In order to acheive appropriate gene activation, it is probable that receptors must interact with other transcription factors in the promoter region. How the specific cellular effects of these hormones are determined is not well understood. The relative levels of hormones as well as differential expression of their respective receptors in the cell may play a role. If more than one class of receptor is present, then their relative levels will be important if they compete for a common target sequence. It is also possible that receptors that recognize the same response element may have different binding affinities or different classes of receptor may differ in their ability to interact with particular transcription factor(s). Finally, the precise arrangement of ERE or PRE and other promoter elements could influence the sensitivity of a promoter to a particular hormone. As already mentioned, PR forms A and B have different promoter specificities and may have different potential to synergize or antagonize with other upstream elements or factors involved in modulation of transcription.

13 SUMMARY AND HYPOTHESES

OT is widely used to induce and augment labour. Although OT induced contractions mimic physiological labour, the role of OT in the initiation of parturition remains controversial. After critical examination of human and animal studies, the balance of evidence, as reviewed in this chapter, suggests that OT plays a facilitatory role during the second and third stages of labour but not in the process of the initiation of parturition.

We and others have proposed that human labour may be regulated by factors synthesized within intrauterine tissues. Amnion, chorion and decidua form a paracrine network with substantial capacity to synthesize various peptides, steroids and prostanoids, immediately adjacent to the likely target of these compounds, the myometrium. We have proposed an extension to this paracrine network by hypothesizing that OT is synthesized locally in these tissues. Thus, changes in OT levels at the time of parturition may occur without affecting maternal circulating levels. Such a finding could essentially negate the previous conclusions about the role of OT in human parturition based on measurements of peripheral concentrations of OT.

In several animal species, circulating levels of estradiol increase prior to labour onset. Although no such changes in plasma levels occur in humans, the capacity of chorion and decidua to synthesize estradiol increases around the time of parturition. Estrogen increases the responsiveness of the myometrium by inducing OT receptor synthesis and stimulating gap junction formation leading to increased electrical coupling between myometrial cells. These changes result in coordinated uterine contractions at the time of parturition.

There also is evidence that estrogen can stimulate OT synthesis in the human. Higher plasma levels of estradiol during the menstrual cycle or with use of oral contraceptives are associated with increased circulating OT levels. Furthermore, estradiol and progesterone receptors are present in human intrauterine tissues, suggesting that estrogen may have a physiologic role in these tissues.

These data led us to the following hypotheses:

1. **OT is synthesized in human amnion, chorion and decidua and the rate of synthesis increases at parturition.**

2. **OT mRNA synthesis in amnion, chorion and decidua is regulated by estrogen and progesterone.**

3. The estrogen receptor gene is expressed in amnion, chorion and decidua and its synthesis is increased at parturition.

The following chapters (IV and V) contain two manuscripts describing our experiments that addressed these hypotheses.

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CHAPTER IV

SYNTHESIS OF OXYTOCIN IN AMNION, CHORION AND DECIDUA MAY INFLUENCE THE TIMING OF HUMAN PARTURITION

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INTRODUCTION

Parturition in most mammalian species is preceded by a rise in estrogen and a decline in progesterone concentrations in maternal plasma (1). These events occur over several hours or days prior to parturition and lead to changes that increase myometrial contractility. These changes include an increase in myometrial oxytocin (OT) receptors and gap junction formation (2,3). Immediately prior to labor onset, neurohypophyseal OT is released into the maternal circulation (4). This release is accompanied by increased production of prostaglandins from intrauterine tissues (5). The end result is coordinated uterine contractions resulting in delivery of the fetus.

In the human, the mechanisms regulating the timing of parturition are poorly understood. Most investigators have failed to show any significant changes in maternal plasma concentrations of estrogen, progesterone or OT preceding labor onset (6). We and others have hypothesized that the basic mechanisms underlying parturition may be similar in the human and other mammals but, in the human, events may occur in a paracrine fashion within intrauterine tissues, particularly within the amnionic and chorionic membranes and the maternal decidua. Thus, local alterations in the levels of paracrine regulatory factors could occur without being reflected in the maternal circulation. In this regard, we have demonstrated synthesis of estrogen and progesterone within intrauterine tissues and have shown a significant increase in the estrogen/progesterone ratio around the time of parturition (7,8,9). These tissues are also the sites of synthesis of prostaglandins that enhance myometrial contractions in labor (10,11).

Despite the widespread use of OT to induce or augment human labor, there is considerable doubt about the physiologic role of OT in normal human parturition. This doubt is based on the following findings: (a) most investigators have been unable to detect an increase in plasma OT prior to labor onset (12,13); (b) there is no apparent correlation between plasma OT levels and myometrial activity (14); (c) plasma OT concentrations at labor onset are 10-1000 fold lower than the K_d of the myometrial OT receptors (15); and (d) human labor occurs normally in cases of maternal posterior pituitary dysfunction and in the absence of OT from the fetal circulation (as in fetal anencephaly) (16). On the basis of this dichotomy, we hypothesized that, like the steroid hormones, local synthesis of OT within intrauterine tissues in late human gestation may play an important role in regulating parturition. This would rationalize the clinical observations regarding the physiologic nature of OT-stimulated labor with the evidence suggesting that OT does not regulate the timing of labor onset in an endocrine fashion.

In this paper, we have studied OT gene expression in amnion, chorion laeve and decidua around the time of parturition. The amnion and chorion are derived from fetal tissues. The amnion consists of a single layer of cuboidal epithelial cells on a loose connective tissue matrix. It is in immediate contact with the amniotic fluid on one surface and the underlying chorion on the other. The chorion laeve is an extension of trophoblast cells around the entire uterine cavity. Except at the placental site, it is intimately in contact with the maternal decidua which, in turn, is contiguous with the myometrium. Thus, this potential paracrine unit is ideally situated to mediate signals of fetal or maternal origin to the pregnant myometrium and hence play an important role in the timing of parturition. Our experiments demonstrate, using Northern blot analysis, ribonuclease protection assays and *in situ* hybridization, that OT mRNA is synthesized within intrauterine tissues. Furthermore OT gene expression is increased around the time of labor onset and a similar increase can be stimulated *in vitro* by estradiol. These findings support our hypothesis of a paracrine system involving OT and sex steroids that may stimulate myometrial contractions in the absence of changes in maternal plasma concentrations.

MATERIALS AND METHODS

Tissue preparation

Following approval from the Human Ethics Review Board, tissues were obtained from uncomplicated pregnancies at term immediately following spontaneous labor and delivery or at repeat cesarean section prior to labor. The amnion is easily separated from the chorio-decidua and is essentially free of contamination with other cell types. The adherent decidua is carefully dissected from the chorion. On histologic examination, the decidua has very little contamination with chorion cells but the chorion may contain up to 20% decidual cells(17). After separation, the tissues, along with a sample of placental tissue, were immediately frozen in liquid nitrogen or processed for *in situ* hybridization. Corpora lutea were obtained from two patients who underwent ovariectomy. Human pituitaries (females of reproductive age) were removed and frozen in liquid nitrogen at the time of post-mortem. Care was taken not to include any hypothalamic tissue in the sample.

Tissue Explant Cultures

Using a sterile cork borer, 2.5 cm. discs of unseparated amnion and chorio-decidual tissue were excised and incubated in "pseudo-amniotic fluid" (18) at 37^0 C in 95:5 air:CO_2 for 12 hours. This medium consists of electrolytes, glucose, urea and albumin in concentrations similar to human amniotic fluid. Estradiol was added to the medium in concentrations of 0, 0.1 and 1.0 nmol/L. We have chosen this culture system because it allows study of the intact tissues in their own matrix and avoids the potentially artifact-producing procedures of cell dispersion and culture. In this system, the cells remain metabolically, endocrinologically and histologically intact for at least five days (17).

Northern Blot Analysis

Total RNA was isolated from the human tissues by the guanidine isothiocyanate/cesium chloride gradient technique and poly A^+ RNA was obtained using oligo dT cellulose columns (19). Five µg of poly A^+ RNA was fractionated on 1.5 % agarose gel in the presence of 1.0 M formaldehyde and transferred to nitrocellulose filters (20). The 3'-specific human OT cDNA probe (21) used in this study is 187 bp in length, including a 30 bp linker. It corresponds to the final 60 nucleotides of exon 3 coding for the carboxy terminal end of the neurophysin-I molecule and the adjacent sequences of the 3' untranslated region. This is the area with least homology with the vasopressin gene and renders the probe specific for the oxytocin gene. The rat hypothalamic RNA was obtained and the antisense OT RNA probes were prepared as previously described (22) with SP6 RNA polymerase (Promega Bio) and [^{32}P]-CTP (New England Nuclear, Boston, MA, 800 Ci/mmol) under conditions described by Melton et al (23). The antisense RNA probes were hybridized to the immobilized RNA in the presence of 50% formamide, 50 mM PIPES (pH 6.8), 5X Denhardt's, 0.1% SDS, 100 mg salmon sperm and herring sperm DNA and yeast tRNA at 65^0 C for 12-16 hours (22). Filters were subsequently washed to a stringency of 0.05 X SSC at 65^0 C. Nitrocellulose filters were exposed to XAR or XRP X-ray films

(Kodak) for 1-7 days. To confirm that equivalent amounts of RNA were loaded on each lane, blots were reprobed with [^{32}P] labelled γ-actin cRNA probes. The γ-actin probe consists of 300 nucleotides of distal 3'-untranslated region of the cDNA clone pHFgA (24).

In situ Hybridization

Tissues were immersion fixed, cryoprotected and sectioned onto chromalum subbed slides. After fixation in phosphate buffer containing 4% paraformaldehyde for 20 minutes and treatment with proteinase K, sections were prehybridized and hybridized with the [^{35}S]-labelled 3'-specific OT antisense cRNA probe at 42^{0} C (22). The hybridization solution was similar to that used for Northern blot analysis except for the presence of 20 mM DTT. Following RNase treatment (200 mg/ml) and washings up to 0.1% SSC at 42^{0} C, slides were exposed to XAR X-ray film for 1-2 days to determine the optimum time of exposure with NTB-2. Slides were dipped in NTB-2 emulsion and exposed for 2-5 days at 4^{0} C. Sections were developed, counterstained with hematoxylin and eosin and analysed by darkfield and brightfield microscopy. Control slides were incubated with sense cRNA probe of similar specific activity.

Ribonuclease Protection Assays

Total RNA from various tissues was isolated as described (19). The ribonuclease protection assays were performed using a modification of a previously published technique (19). Total RNA (70-100 µg) was hybridized to gel-purified human OT antisense ^{32}P-labelled cRNA probe in 80% formamide and 20% PIPES for 16 hours at

55^0 C. Template DNA was removed from the probe by treatment with ribonuclease-free DNase. Following incubation with ribonuclease A (2.5 µg/ml) and T_1 (330 U/ml), protected fragments were analyzed on 6% denaturing polyacylamide gels. The gel was exposed to XAR X-ray film with an intensifying screen for 1-7 days.

Following extraction of RNA from the tissues, stock solutions were made for each sample and the concentration of RNA measured by spectrophotometry. For each gel, equal amounts of total RNA were used for each sample To confirm that equal amounts of RNA were applied, in some experiments, the protection assays were performed using a probe to γ-actin, a constitutive component of these tissues which we assume to be similarly abundant in the fetal membrane tissues and not to change with parturition. Since this mRNA is more abundant than the OT mRNA, smaller aliquots of RNA were necessary. Ten µg of total RNA was used with the γ-actin probe. In these experiments the γ-actin probe corresponds to 270 nucleotides of the C-terminal coding region, 130 nucleotides of the 3' untranslated region and 130 nucleotides of vector sequences.

The 5' human OT probe used in these studies consists of approximately 360 nucleotides corresponding to the OT peptide coding region of exon 1 and all of exon 2.

For comparing amounts of OT mRNA between the cesarean section and spontaneous labor groups and for assessing the response to estrogen, laser densitometry was used to give quantitative data.

Because of the heterogeneous variance in the small number of samples, statistical comparisons were performed using the Mann-Whitney U test with statistical significance being defined as a p value < 0.05.

RESULTS

To determine whether oxytocin (OT) mRNA is synthesized in intrauterine tissues, amnion, chorion and decidua were dissected and RNA was isolated. OT mRNA was present in all three of these tissues (Fig. 1a.). Levels were highest in maternal decidua, and were approximately equal in chorion and amnion. We also examined a number of other human tissues and cell lines. OT mRNA was also expressed in human ovary, as previously described (25), as well as in the pituitary and placenta, although levels were much lower than in the intrauterine tissues (Fig. 1a). No OT mRNA was detected in the human 4A neuroblastoma cell line or in rat cerebral cortex, even upon prolonged exposures. Hybridization of the same Northern blot to a probe specific for γ-actin mRNA indicated that approximately equal amounts of poly A^+ RNA were present in each lane (Fig. 1b). Using the 3' specific probe for human g actin, no hybridization was detected in either the rat or human neural tissues.

The cellular localization of OT mRNA was performed by *in situ* hybridization (Fig. 2). Amnion, chorion and decidua all demonstrated hybridization to the antisense cRNA probe (Fig. 2c; for histology, see Fig. 2a). The epithelial layer of amnion demonstrated a small amount of specific hybridization. The chorionic membrane, originating from the outer cell mass of the blastocyst, consists of connective tissue and a layer of trophoblast 4-10 cells thick. In contrast to the very low levels of hybridization in the placental trophoblast (data not shown), the chorionic trophoblast shows abundant silver grains denoting presence of OT mRNA. The maternally-derived decidua demonstrated the highest levels of hybridization which appeared to be concentrated in the deeper layers lying nearest the myometrium. It is not possible with our current histology to identify with certainty the specific cell types synthesizing OT mRNA. Control incubations using the sense probe demonstrated only minimal background (Fig. 2b).

To confirm these findings and to quantitate the relative levels of OT mRNA in different tissues, ribonuclease protection assays were performed (Fig. 3). Hybridization of 100 mg of total RNA to a [^{32}P]-labelled OT cRNA probe produced a protected band of approximately 160 nucleotides, the expected size, in amnion, chorion, decidua, and placenta (Fig. 3a). As with Northern blot analysis, the decidua expressed greater levels of OT mRNA than either amnion or chorion. Specific hybridization to placental and ovarian mRNA (Fig. 3a) was detected only after prolonged exposures. No protected band was detected following hybridization of the OT cRNA probe to tRNA or to total RNA isolated from rat hypothalamus, indicating that even the 85% homology between the human and rat mRNAs was not sufficient to protect the human probe in this assay. To ensure the validity of these quantitative comparisons, ribonuclease protection assays were performed with a probe specific for γ-actin mRNA. Approximately equal amounts of γ-actin mRNA were present in the amnion, chorion, decidua and placenta whereas there was somewhat less RNA in the ovary lane (Fig. 3c).

Ribonuclease protection assays also were performed with a 5' probe that included the coding region for the OT peptide. A protected fragment was detected in total RNA from the chorio-decidua (Fig. 3b), indicating that the OT mRNA detected in this tissue could produce the biologically active peptide.

These data suggest that locally synthesized OT may participate in a paracrine mechanism regulating the timing of labor onset. To test this hypothesis, we isolated total RNA from chorio-decidua obtained at elective repeat cesarean section (CS) prior to labor (38.4 ± 0.9 weeks gestation, standard error of the mean, (S.E.M.)) or following spontaneous onset of labor (SL) and vaginal delivery (40.3 ± 0.6) and determined levels of OT mRNA. Ribonuclease protection assays of equal amounts of total RNA from 7 pre-labor and 6 post-labor samples revealed that OT mRNA levels were consistently higher in the chorio-decidua following parturition (Fig. 4a, b). Scanning laser densitometry revealed that SL tissues contained 3-4-fold higher levels of OT mRNA than did CS tissues (Fig. 4a;1.8 ± 0.2 vs 0.6 ± 0.1 units, $p < 0.008$ and Fig. 4b; 3.0 ± 0.5 vs 0.8 ± 0.7 units, $p < 0.04$). Similarly, when levels of OT mRNA were normalized by comparison with levels of γ-actin mRNA in the same samples (Fig. 4c), as determined by ribonuclease protection assays, the ratios of OT/γ-actin were significantly higher in SL compared to CS tissues ($p < 0.03$). OT mRNA levels were similar in all of the different post-labor chorio-decidua samples, but were somewhat variable in pre-labor samples, posssibly due to differences in the proximity to the time when spontaneous labor would have occurred in the elective cesarean section patient population.

One potential mediator of this increase in OT mRNA observed in the post-labor samples is estradiol. We have previously demonstrated that estradiol biosynthesis increases in human fetal membranes around the time of parturition (7). Estradiol increases synthesis and release of OT from the neurohypophyseal system (20,26,27). To determine whether estradiol can increase OT mRNA levels in chorio-decidua, we incubated full thickness membrane explants (amnion, chorion, and decidua) in the presence of various concentrations of estradiol for 12 hours. Ribonuclease protection assays of equal amounts of total RNA isolated from these explants indicated that estradiol (0.1 and 1.0 nmol/L) increased OT mRNA content relative to controls incubated for the same period of time with no estradiol (Fig. 5).

DISCUSSION

The results of this study demonstrate a new peripheral site of OT synthesis, the human intrauterine tissues of both fetal and maternal origin. The fetal membranes and maternal decidua are believed to play an important role in the metabolic events leading to the initiation of parturition (28). The juxtaposition of these tissues next to the myometrium situates them ideally to influence myometrial contractility. The demonstration of a three- to four-fold increase in OT gene expression following labor and delivery and its regulation *in vitro* by estradiol strengthens our hypothesis that local synthesis of OT could stimulate uterine contractions in a paracrine fashion without increasing circulating maternal OT concentrations.

The Northern blot and ribonuclease protection analyses using the 3'-directed probe indicate that OT mRNA is synthesized predominantly in decidua, with much lower levels in chorion and amnion. We cannot determine what proportion of the chorion OT mRNA may be due to contamination with decidual cells, but *in situ* hybridization demonstrated some OT mRNA in the trophoblast layers of the chorion. The *in situ* hybridization experiments also suggested that decidual cells lying next to the underlying myometrium expressed higher OT mRNA levels than did cells lying closer to the chorion. Thus, the highest local levels of OT synthesis may occur in the region immediately adjacent to the putative target organ, the myometrium.

Recently, it has been demonstrated that mRNA derived from the rat testis vasopressin gene is non-translatable and corresponds to exons 2 and 3 without exon 1, the exon coding for the vasopressin peptide (29). To ensure that exon 1 of the OT gene was transcribed in fetal membrane tissues, we performed ribonuclease protection assays to demonstrate hybridization to the 5' OT probe. The results (Fig. 3b) prove that these tissues transcribe the regions of the OT gene that codes for the OT peptide and rule out the possibility of splice variation of OT mRNA.

An interesting observation in our study was the difference in size of OT transcripts from different intrauterine tissues. Chorion appeared to produce both of the transcripts although we cannot exclude the possibility that the smaller of the two is due to contamination of the chorion tissue with adherent decidua. Decidual OT mRNA is 60-80 nucleotides smaller than that synthesized by chorion, amnion, ovary and placenta. In the chorio-decidua, this difference is not likely due to differences in the coding region of the mRNA since both the 5' and 3' specific probes were completely protected by RNA from these tissues. The difference in size may be due to a variation in the poly(A) tail length, as has previously been noted in bovine hypothalamic and ovarian OT mRNA (30). Differences in poly(A) tail length may provide a mechanism for increasing OT mRNA stability (31,32) and hence, synthesis during times of increasing physiological requirement for this hormone. Support for this hypothesis derives from studies demonstrating that OT mRNA poly(A) tail length increased during pregnancy and lactation (33) and in response to osmotic stimuli (34). The two sizes of transcripts noted in our studies may thus indicate tissue-specific differences in post-transcriptional regulation of OT synthesis.

Our results also demonstrate that OT mRNA is present in the human pituitary and corpus luteum thus supporting previous studies in mammals (35,36,37,38). The human pituitary transcript was smaller in size than the amnion and ovarian transcript. In this regard, Mohr et al (38) demonstrated that OT transcripts found in rat posterior pituitary were smaller than those found in the hypothalamus. It is not known whether OT is synthesized in the human pituitary or transported axonally from the hypothalamus as in the rat (39).

Comparison of pre-labor and post-labor tissues revealed that OT mRNA levels were higher following the spontaneous onset of labor. We have used ribonuclease protection assays to make these comparisons not only because of the specificity of these assays but also because we feel that the solution hybridization step provides a better basis for quantification than Northern blot analysis. Quantification by laser densitometry revealed that the 3-4-fold increase in OT mRNA seen in the tissues following the spontaneous onset of labor was statistically significant. The OT mRNA levels tended to be similar in all post-labor chorio-decidua samples, suggesting that a maximal level of gene expression had occurred.

However, there was considerable variability in the pre-labor samples which may be due to the fact that these samples were obtained at varying intervals prior to the time when spontaneous labor would have occurred. Although we cannot determine if the increase in OT gene transcription was a cause or result of labor, this finding is consistent with the hypothesis that increased local synthesis of OT may play a role in the physiology of human parturition. A recent study (40) has demonstrated an increase in pulsatile bursts of OT in the maternal circulation prior to spontaneous labour. However, the origin of maternal circulatory OT is not known nor is its role in the initiation of labour.

The data of Fig. 5 suggest that estradiol may regulate OT gene expression in fetal membranes. Previous studies have demonstrated that an increase in plasma estradiol at the time of parturition (1) and sexual maturation stimulates hypothalamic OT synthesis and release in the rat (19). Furthermore, the human OT promoter contains a functional estradiol responsive element (41). Human amnion, chorion and decidua are all steroidogenic tissues that produce estrogen and progesterone primarily from sulfurylated precursors (7). We have demonstrated that the rate of estrogen production and the local estrogen/progesterone ratio increases in fetal membranes around the time of parturition (9). In addition, estradiol receptors are present in decidua and increase in response to treatment with the progesterone receptor antagonist RU486 (42). Thus, our results demonstrating that estradiol increased OT gene expression in amnion-chorion-decidua explant cultures may have physiologic relevance.

Estradiol is not the only factor that is likely to regulate intrauterine OT biosynthesis. Several studies suggest an interrelationship between OT and prostaglandins. Stimulatory prostaglandins are synthesized within decidua and amnion, and their production increases around the time of labor onset (43,44). Oxytocin stimulates prostaglandin synthesis from decidua and amnion, and this stimulatory activity is greater in tissues collected following labor than prior to labor onset (45,46,47). Decidual and myometrial cells also contain OT receptors that increase greatly around the time of parturition (48,49,50). The resultant increased sensitivity of the uterus to OT may be an important step not only in the second stage of labor, when circulating serum concentrations of OT are increased, but also in the initiation of labor. A close temporal relationship between increased OT responsiveness and uterine prostaglandin synthesis has been demonstrated in the rat (46). Induction of labor with OT in women is successful only when OT infusion is associated with an increase in production of PGF_{2a} (51,52). Suppression of prostaglandin synthesis decreases myometrial OT responsiveness as well as OT binding sites (43). These findings support the hypothesis that OT synthesized in decidua may act in an autocrine or paracrine fashion to stimulate intrauterine prostaglandin biosynthesis. In turn, prostaglandins stimulate synthesis of OT receptors and gap junction formation in myometrial cells (53,54).

Inappropriate induction of OT synthesis in fetal membranes may also play an important role in the onset of premature labor. It is possible that locally synthesized OT may be a mediator of the pathophysiologic process causing premature labor following infection of the fetal membranes (55,56). Mediators of the immune system stimulate synthesis and release of OT from the neurohypophysis. Christensen et al (57) demonstrated a dose-dependent effect of interleukin-1b on the *in vitro* release of OT from the rat neurohypophysis. An increase in plasma levels of OT occurs following intravenous injection of lipopolysaccharides (58). Of interest in this regard, the promoter region of the rat OT gene contains an interferon response element (59) suggesting the possibility of direct regulation of the OT gene by the products of the immune system. In addition, cytokines (interleukins, tumor necrosis factor, lipopolysaccharides) stimulate prostaglandin biosynthesis from intrauterine tissues (60,61,62,63). Early induction of local OT synthesis in fetal membranes by any of these factors may have important consequences for the timing of parturition.

In conclusion, our findings suggest a potentially important role for OT in the physiology of human parturition. They also rationalize how this hormone, with such specific and potent ability to stimulate human myometrial contractions, may play a pivotal role in labor onset in the absence of significant changes in maternal plasma concentrations. The results are compatible with the hypothesis of an intrauterine paracrine system with positive interactions among oxytocin, estradiol and prostaglandins. The local increase in the estrogen/progesterone ratio in late pregnancy may activate OT gene transcription and increase the number of OT receptors in myometrium and decidua. The latter may sensitize both the myometrium and the decidua to any local OT, thereby leading to increasing contractile activity and increasing production of stimulatory prostaglandins, respectively. The increased prostaglandins would directly stimulate the myometrium and potentially lead to further increases in production of oxytocin and its receptor. This coordinated interaction involving estradiol, OT and prostaglandins in human fetal membranes could ultimately result in the onset of parturition. Given that premature labor remains the major contributing factor to perinatal mortality and morbidity, and that potent OT antagonists recently have been developed (64,65), this information may be important both in understanding the regulation of human parturition and in preventing preterm delivery. Further studies elucidating regulatory mechanisms controlling OT gene expression in intrauterine tissues may lead to new strategies to prevent or treat premature labor.

FIGURE LEGENDS

Figure 1. a; Northern blot analysis of OT mRNA in human tissues: RH, rat hypothalamus (positive control); CD, chorio-decidua; CH, chorion; DE, decidua; AM, amnion; PL, placenta; OV, ovary; PI, pituitary; NE, human neuroblastoma 4A cell line (negative control) and RC, rat cortex. b; Hybridization of the same blot with human γ-actin probe to demonstrate the relative amounts of RNA in each lane from the CD to OV lanes. PL* is a 7-day exposure of the placental lane compared to a 36-hour exposure for the other lanes. The predominant transcript in decidua is 60-80 nucleotides smaller than the transcript seen in amnion, ovary and placenta.

Figure IV-1.

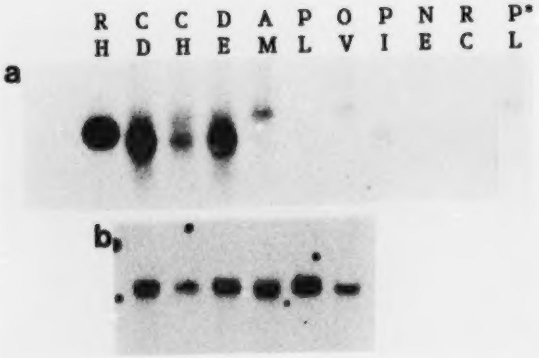


Figure 2. *In situ* hybridization for OT mRNA in human intrauterine tissues. a; Brightfield photomicrographs of human amnion (A), chorion (C) and decidua (D) to demonstrate the histology of the tissues. b; Darkfield photomicrograph using an antisense cRNA probe demonstrating the specific hybridization to OT mRNA in amnion, chorion and decidua. c; Darkfield photomicrograph of human amnion, chorion and decidua hybridized with a labelled sense cRNA probe to act as a control for (b). Specific activity of the probe and photographic conditions are similar to that for b. All photomicrographs are at the same magnification. Bar = 20 mm.

Figure IV-2.

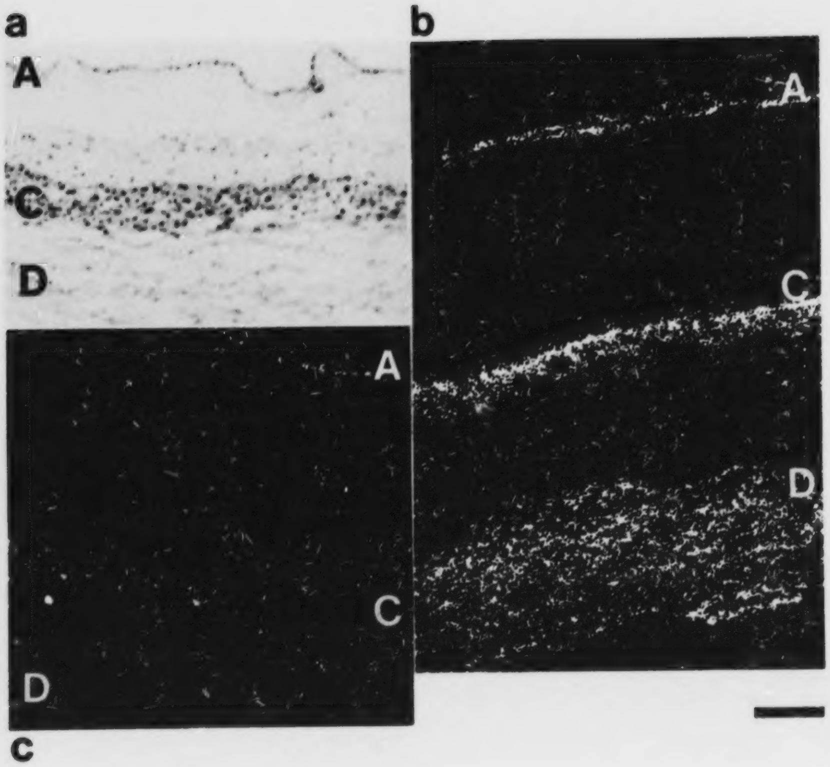


Figure 3. Ribonuclease protection assay for OT mRNA in human tissues using a 3'-directed probe specific for the OT gene. a; samples hybridized with a 3'-specific probe. Lanes correspond to the probe (PR), amnion (A), chorion (C), decidua (D), placenta (P) and ovary (O). Negative controls are rat hypothalamus (R) and tRNA (T). Protected bands were not observed in the placental and ovarian samples at this exposure time: prolonged exposure times were required to detect positive signals in these tissues. b; Ribonuclease protection assay using a 5' specific probe corresponding to parts of exons 1 and 2 coding for the OT peptide and the amino-terminal end of neurophysin I. The lanes contain the probe (PR), tRNA (T) used as a negative control and a sample of chorio-decidua (CD) obtained following the spontaneous onset of labor. The lanes adjacent to CD contain a small amount of RNA that has spilled from lane CD. c; Ribonuclease protection assay performed with a γ-actin probe to demonstrate the amounts of RNA in each of the sample lanes in (a). Relatively equal amounts of RNA are present in the lanes containing samples A, C, D and P whereas the lane containing the sample from human ovary has somewhat less RNA. The human probe was not protected by rat hypothalamic RNA or tRNA.

Figure IV-3.

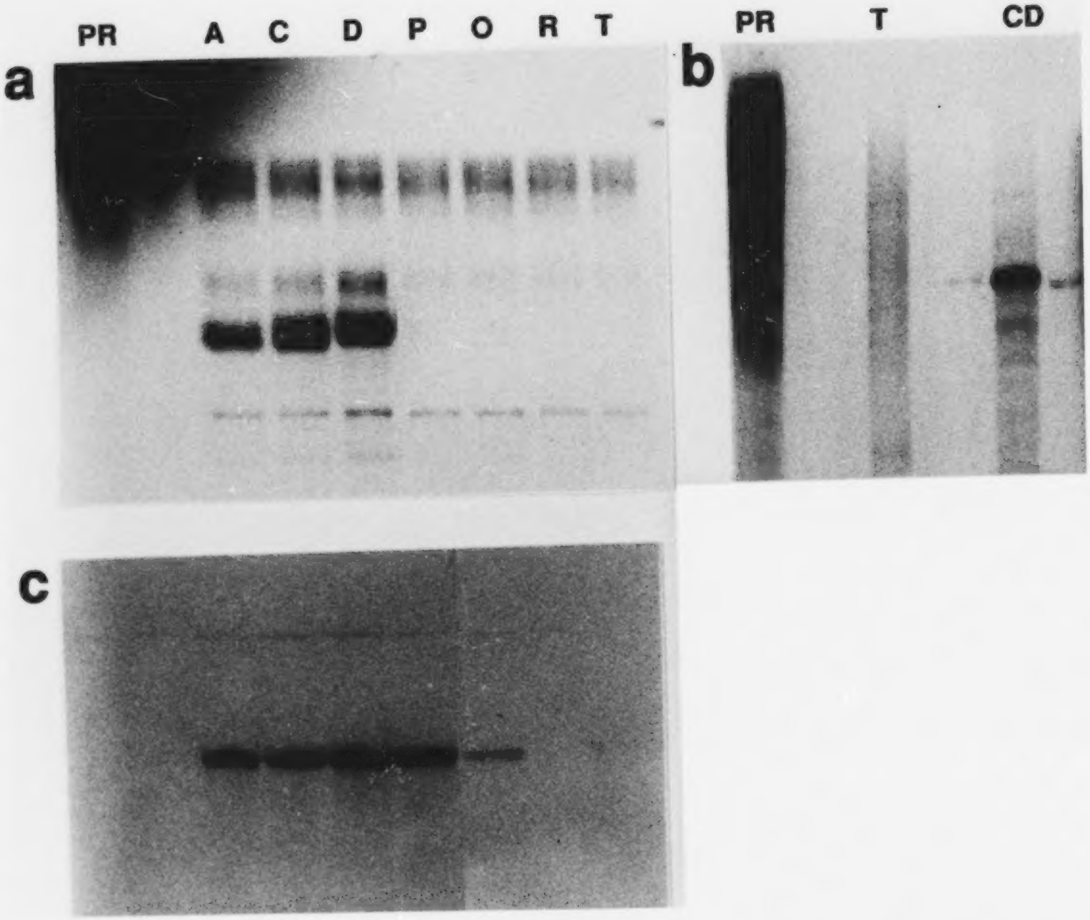


Figure 4. Ribonuclease protection assays using RNA from human chorio-decidua collected at elective cesarean section (CS) at term but prior to labor onset or at term following the spontaneous onset of labor (SL) and vaginal delivery. a; three separate samples from each of the CS and SL groups Equivalent amounts of total RNA were applied to each lane and the samples were run on the same gel. For the sake of clarity, the pictures have been spliced to eliminate empty lanes. b; similar to (a) showing several additional and different samples c; the same samples as (b) but hybridized to a γ-actin probe to show relative amounts of RNA in each lane. In all panels, P identifies the undigested probe and MW indicates the DNA molecular weight standards (220 and 152 nucleotides for (a) and (b); 396 and 344 nucleotides for (c)).

Figure IV-4.

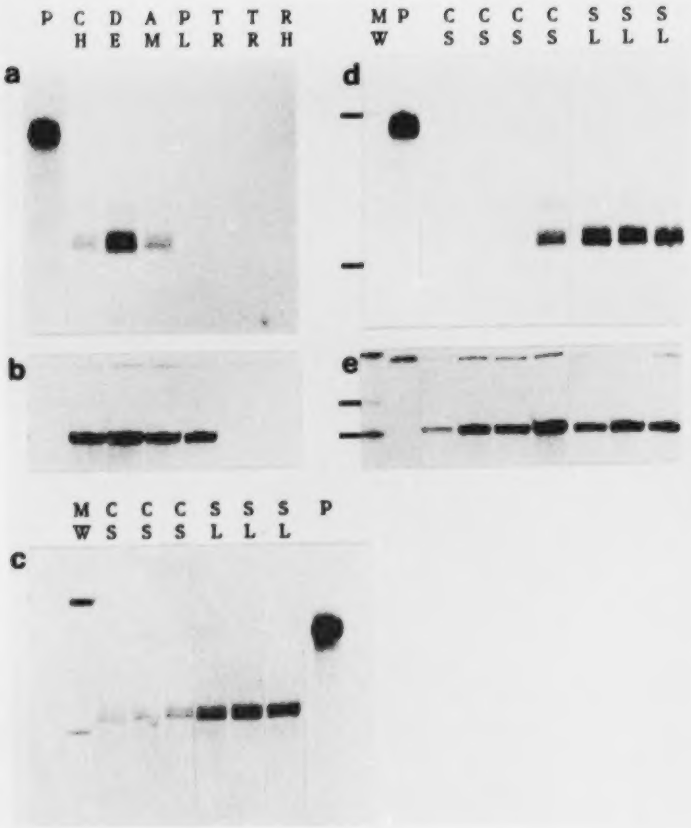
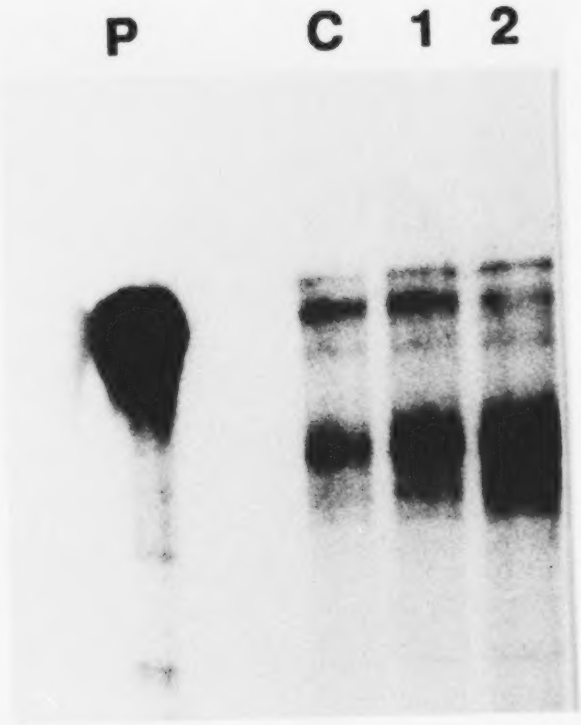

P CH DE AM PL TR TR RH
a
b
MW P CS CS CS CS SL SL SL
d
e
MW CS CS CS SL SL SL P
c

Figure 5. Ribonuclease protection assay for OT mRNA from explant cultures of full thickness human amnion and chorio-decidua incubated in the presence or absence of estradiol. The tissues were obtained at elective cesarean section and incubated for 12h in pseudo-amniotic fluid. The lanes represent the probe (P) or RNA extracted from the tissues incubated in the absence (C) or presence of 0.1 and 1.0 nmol/L estradiol (lanes 1 and 2 respectively).

Figure IV-5.



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CHAPTER V

REGULATION OF OXYTOCIN GENE EXPRESSION IN HUMAN FETAL MEMBRANES AND DECIDUA BY STEROID HORMONES.

A version of this chapter is being prepared as a manuscript for submission to *The Journal of Clinical Investigation*.

INTRODUCTION

The mechanisms regulating the timing of human parturition are poorly understood. Premature birth accounts for 70-85 % of all perinatal deaths and for the major proportion of mental and physical handicaps resulting from the birth process. Stratagies to prevent or treat premature labor have largely been unsuccessful. Better results depend upon a clearer understanding of the physiology of labor onset. Oxytocin (OT) is a potent and specific uterotonic agent that is widely used to augment or induce labor. However, the physiological role of OT in the initiation of parturition remains controversial (1-3). This controversy is based largely on the failure to demonstrate increases in plasma concentrations of OT prior to the onset of labor. Several investigators have suggested that the signaling pathway for the initiation of human labor involves autocrine or paracrine mechanisms within intrauterine tissues, particularly the amnion and chorion membranes and the maternal decidua (3-5). Recently, we demonstrated that OT mRNA is synthesized in human amnion, chorion and decidua and that there is a significant increase in OT mRNA levels in chorio-decidua in association with parturition (6). We hypothesized that this increase in local synthesis of OT may influence the timing of labor onset in a paracrine fashion.

In several animal models, estrogen and progesterone play an important role in the maintenance of pregnancy and initiation of labor. In these species, the onset of labor is preceded by an increase in estrogen and decrease in progesterone concentrations in maternal peripheral plasma (7,8). Previously, we have demonstrated that estrogen is synthesized within intrauterine tissues and this increases around the time of parturition (9,10). At the same time there is an increase in metabolism of progesterone in fetal membranes (9). The resultant increase in the local estrogen/progesterone ratio may have several actions on the myometrium that prepare the uterus for parturition. Estrogens increase synthesis of the contractile proteins actin and myosin that are necessary for muscle activity (11). Estrogens also increase OT receptor concentrations and gap junction formation in myometrial cells (12-14). These changes sensitize the myometrium to OT and synchronize uterine contractions.

Estrogen also increases OT synthesis and secretion from the hypothalamo-neurohypophysial system (15,16) and preovulatory ovarian follicles (17). In support of a direct effect of estradiol on OT gene expression, transfection studies have demonstrated that both the rat and human OT promoter contain a functional estrogen response element (18). Estradiol receptors (E_R) are present in human decidua at term, although no changes in E_R numbers have been reported prior to or following parturition (19,20). Receptors for estrogen and progesterone have not been detected on amnion and chorion (20,21).

In the present studies, we explored the possibility that changes in estrogen and progesterone may regulate OT gene expression in human amnion/chorion/decidua at term. Additionally we determined the effects of estrogen receptor blockade on OT mRNA synthesis and measured mRNA levels for estrogen and progesterone receptors in intrauterine tissues.

MATERIALS AND METHODS

Tissue Preparation

Tissue collection was approved by the Research Ethics Board of the University of Alberta. All tissues were obtained from uncomplicated pregnancies at term immediately following spontaneous labor and delivery or at repeat cesarean section prior to labor onset. Using a sterile cork borer, 2.5 cm discs of full thickness (unseparated amnion, chorio-decidual tissue) were excised and incubated in 50 mL of pseudo-amniotic fluid (22) for varying times at 37° C in 95:5 air:CO_2. The medium then was changed and incubations continued in the absence or presence of the potential regulators. Estradiol was added in final concentrations of 0.1, 1, 5, 10 and 100 nmol/L. For progesterone and tamoxifen, the final concentrations were 0.1, 1 and 100 μmol/L. Cycloheximide (1-100 μmol/L), α-amanitin (50-100 nmol/L) and actinomycin D (1-5 μmol/L) were added to determine the role of protein or RNA synthesis in any of the changes observed. All incubations were performed in triplicate.

This culture system allows the study of intact tissues with their matrix retained and avoids the potentially artifact-producing procedures of cell dispersion and culture. In this system, the cells remain metabolically, endocrinologically and histologically intact for at least five days (23). At the end of the incubation period, tissues were frozen in liquid nitrogen and processed for RNA isolation.

Hybridization probes

The specific human OT cDNA probe (24) used in this study is 187 nucleotides in length, including a 30 nucleotide linker region. It corresponds to the final 60 nucleotides of exon 3 coding for the carboxy terminal end of the neurophysin-I molecule and the adjacent sequences of the 3' untranslated region. This is the area with least homology with the vasopressin gene and renders the probe specific for the OT gene. The E_R cDNA (25) corresponds to the DNA binding domain and is 330 nucleotides in length including 15 nucleotides of the multiple cloning site. The progesterone receptor (P_R) cDNA (26) contains 360 nucleotides of C-terminal coding sequences encoding the hormone binding domain, 40 nucleotides of the adjacent 3' untranslated region and 35 nucleotides of the multiple cloning site. The γ-actin probe (27) corresponds to 270 nucleotides of the C-terminal coding region, 130 nucleotides of the 3' untranslated region and 130 nucleotides of vector sequences. Antisense OT RNA probes were prepared as previously described (6) with SP6 or T_7 RNA polymerase (Promega Biotech) and [^{32}P]-CTP (New England Nuclear, Boston, MA, 800 Ci/mmol).

Ribonuclease Protection Assays

Total RNA was isolated from the tissues by the guanidine isothiocyanate/cesium chloride gradient technique as described earlier. Total RNA (100 µg) was hybridized to DNase treated, gel-purified antisense ^{32}P-labelled OT cRNA probe in 80% formamide and 5 x salts for 16 h at 55° C (6). Following incubation with RNase A (2.5 µg/ml) and T_1 (330 U/ml) for 30 min at 30° C, protected fragments were analyzed on 6 or 7% denaturing polyacrylamide gels. The gel was exposed to XAR x-ray film with an intensifying screen for 48-72 h. Ten or 25 µg of total RNA was used with γ-actin and E_R or P_R probes. Seventy-five to 100 µg RNA was used with the OT probe. RNA concentrations were measured by spectrophotometry. Equal amounts of RNA were applied to each lane and this was confirmed using the γ-actin probe as an internal standard. Autoradiograms were quantitated using the LKB Ultrascan XL scanning laser densitometer.

RESULTS

To determine the optimal time for incubation of explants, we isolated total RNA at different time points and analysed OT mRNA using ribonulease protection assays. The analyses demonstrated that OT mRNA levels were increased at 1 and 4 hours relative to time 0 and declined with longer incubations (12, 24 and 72 hours). The higher levels of OT mRNA observed at 1 and 4 h may be due to non-specific stimulation of OT gene expression resulting from the presence of blood elements and/or from endogenous estrogen or other stimulatory substances. Therefore, for further experiments, the cultures were incubated for 12 hours to allow OT mRNA to reach baseline levels, and treatments were then initiated for a further 12-16 hours.

To determine the effect of estradiol on OT gene expression, total RNA was isolated following incubation of explants with 1, 10, 100 and 1000 nmol/L estradiol for 16 hours. At 1 and 10 nmol/L, an x-fold increase was observed compared to control values (Fig. 1a,b.). Higher concentrations of estradiol (100 and 1000 nmol/L) resulted in OT mRNA levels similar to or slightly higher than respective controls without estradiol but lower than explants treated with 1 and 10 nmol/L of estradiol.

To determine the effects of progesterone on OT gene expression, we incubated explants with 1 and 100 µmol/L progesterone for 12 h (Fig. 1b.). The levels of OT mRNA were not affected by progesterone treatment. To determine whether progesterone influences the effects of estradiol on OT gene expression, we co-incubated explants with 10 nmol/L estradiol and 10 µmol/L progesterone for 12 h. In co-incubation, progesterone had no consistent effect but, in some experiments, partially inhibited the estradiol-induced increase in OT mRNA.

To determine whether the effects of estradiol are receptor mediated, tamoxifen (1.0 and 10 µmol/L), a nonsteroidal estradiol receptor antagonist, alone, or in combination with estradiol was added to the explants for 12 h (Fig. 2). With tamoxifen alone, there was little, if any, effect on the expression of OT mRNA. When combined with estradiol (10 nmol/L estradiol and 10 µmol/L tamoxifen), tamoxifen largely prevented the estradiol-induced increase in OT mRNA. Thus it appears that the effects of estradiol are mediated by estradiol receptors.

Regulation of OT gene expression by estrogen may occur directly at the transcriptional level, at the level of degradation of OT mRNA or, alternatively, may involve induction of a new protein that stimulates OT gene transcription. To further characterize the underlying cellular mechanisms, we investigated whether synthesis of new protein or RNA was required for the estradiol-mediated up-regulation of OT mRNA (Fig. 3.). Explants were incubated with α-amanitin (50 and 100 nmol/L) an inhibitor of RNA synthesis or with cycloheximide (1 and 10 µmol/L), a protein synthesis inhibitor, for 12 hours. When estradiol was added in the presence of α-amanitin, the estrogen-stimulated increase in OT mRNA was inhibited, suggesting the effect of estrogen occurred at the level of transcription. Following incubation with cycloheximide, OT mRNA levels increased 2-3 fold. In co-incubations, estradiol and cycloheximide appeared to have additive or synergistic effects on OT mRNA levels.

The OT gene is expressed in amnion, chorion and decidua. To determine which of these tissues is responsive to estradiol, we measured E_R and P_R gene expression by ribonuclease protection assay using RNA isolated from separated amnion, chorion and decidua. Both E_R and P_R mRNA are expressed in all three tissues as well as in tissue from a uterine leiomyoma, which served as a positive control (Fig. 4). Decidua expressed higher levels of both E_R and P_R mRNA relative to chorion and amnion. Although a single band was observed for E_R, two bands were observed with the P_R probe. The size of these two bands differed by approximately 40 nucleotides.

Since there is an increase in OT mRNA levels in chorio-decidua around the time of parturition, we questioned whether a change in E_R or P_R could be partially responsible for this change. Tissues were obtained from 7 patients following spontaneous labor and delivery at term and compared to 5 tissues obtained at elective repeat cesarean section at term. The amount of E_R mRNA was increased significantly in the tissues collected after labor onset (Fig. 5). The level of P_R mRNA did not change significantly.

DISCUSSION

We have determined the effects of estrogen and progesterone on OT mRNA levels in term fetal membranes and decidua. There are three principle conclusions from our studies: 1) estradiol increases OT mRNA levels in these tissues and progesterone may partially prevent the estradiol-induced increase; 2) the effects of estradiol are receptor-mediated and appear to be directly at the transcriptional level; and 3) estradiol and progesterone receptors are present in amnion, chorion and decidua and ER levels increase significantly around the time of parturition.

An endocrine role for OT in human parturition is controversial. However, a paracrine role for OT is suggested by our finding that decidua, the tissue situated adjacent to the myometrium, expresses OT mRNA, and there is a 4-6 fold increase in OT mRNA in chorio-decidua occurring around the time of parturition. The results of the present studies demonstrate that, at physiological concentrations, estradiol increases OT mRNA levels in intrauterine tissue explants. These findings support our hypothesis that a local increase in the tissue concentration of estradiol around the time of parturition increases OT synthesis and may thereby influence the timing of labor onset.

Our findings are in agreement with previous studies demonstrating that estrogen stimulates OT synthesis and release from the neurohypophysis and ovaries (15-17). Our findings are also supported by recent observations in term pregnant rhesus monkeys where nocturnal elevations in maternal plasma OT and estradiol concentrations are correlated with circadian uterine activity (28-30). Supression of estrogen production in these animals inhibits the increased nocturnal uterine activity (30). Together, these findings suggest that estrogen regulates local OT synthesis in addition to the well-described estrogen stimulation of OT receptors (12,13).

E_R is a ligand dependent transcription factor. The human and rat OT promoter both contain an imperfect estradiol response element (ERE) that is capable of conferring estrogen sensitivity on heterologous reporter gene constructs when transfected into E_R-positive tissue culture cells (18). Our data indicate that E_R and P_R mRNAs are present in amnion, chorion and decidua with highest levels of both in decidua. Furthermore, at the time of parturition, a significant increase in E_R mRNA occurred, an event that may play a role in the preparation of the uterus for labour. E_R and P_R have been detected previously by binding studies and immunocytochemistry in the decidua (19-21). However, in contrast to our data, E_R and P_R were not detected in term amnion and chorion, even after RU 486 treatment (20,21). This discrepancy is likely due to the relatively low sensitivity of the methods previously used.

Interestingly, our data also revealed the presence of two transcripts encoding P_R that may differ in their hormone binding or 3′ untranslated regions. The significance of this finding is not known but these two mRNAs may represent transcripts for the A and B forms of P_R. In breast cancer cells lines, A and B forms of P_R

differ from each other at their 5′ amino terminal region (transactivation domain) by 163 amino acids. It has been suggested that A and B forms may have different functions and may be differentially regulated (31). Further studies are needed but this may in itself be a significant finding as differential regulation of the two forms of P_R may have some functional significance.

The peak concentrations of free estradiol in late human pregnancy plasma are in the range of 30 - 50 nmol/L (32). The stimulatory effects of estradiol on OT gene expression are observed at these physiological concentrations. At supraphysiological concentrations, estradiol had little effect on OT mRNA levels - occasionally there appeared to be either slight stimulation or inhibition. This is similar to the pattern observed in the rat uterus and hypothalamus (33,34). Neither the underlying molecular mechanisms nor the biological or pharmacological significance of this biphasic regulation of E_R are known but a similar pattern of regulation of receptor synthesis is seen with glucocorticoid, retinoic acid and thyroid hormone receptors and their respective ligands (35-37). One possible mechanism for the decrease in OT mRNA observed at higher doses of estradiol may be down-regulation of estradiol receptors as occurs in other reproductive tissues (36-39).

In most animal models, progesterone dominance is present throughout pregnancy and decreases prior to labor onset. Our previous studies with amnion, chorion and decidua have demonstrated a decrease in progesterone synthesis and increase in progesterone metabolism *in vitro*, suggesting a local withdrawal of progesterone in association with parturition (4). The present studies demonstrate that progesterone has little primary effect on OT gene expression but may diminish the estradiol-induced increase in OT mRNA. The inhibitory effect of progesterone may in part be by supression of the estradiol-elicited increase in E_R as demonstrated in rat uterus and hamster uterine decidual cells (42,43). Estradiol and progesterone in combination also shorten the estradiol receptor half life in decidual cells (44). The complexity of interaction between steroids and OT is increased by the observations that OT regulates progesterone and estradiol synthesis in luteal tissue (45,46).

Our results with tamoxifen suggest that the estradiol-stimulated increase in OT mRNA is receptor mediated. In co-incubation with estradiol, tamoxifen, which binds to the E_R and prevents binding of the natural hormone, prevented the estradiol-evoked increase in OT gene expression. However, it is possible that not all of the biological effects of tamoxifen that we observed are mediated by E_R. The decrease in OT mRNA at higher concentrations of tamoxifen may be partially due to inhibition of protein kinase-C (49,50) which is involved in the synthesis and release of OT (51).

In our studies, the inhibition of RNA synthesis prevented the estradiol-induced increase in OT mRNA, implying that the effects of estradiol are at the transcriptional level. In contrast, the protein synthesis inhibitor cycloheximide showed synergistic effects with estradiol on OT mRNA levels suggesting that new protein synthesis is not required for increased transcription of OT gene. In some instances, protein synthesis inhibitors increase levels of mRNA by inhibiting synthesis of repressor proteins that interact with specific regulatory sequences in the promoter of the gene (52). Interestingly, a negative regulatory sequence element which binds a HeLa cell specific potential repressor overlaps with the ERE of the OT gene (53). It is not known whether this repressor sequence plays a role in regulation of OT gene expression or whether a similar repressor is present in the fetal membranes and decidua. Alternatively, cycloheximide may have increased the stability of OT mRNA by post-transcriptional mechanisms, an effect that could have been synergistic with estradiol (54).

In conclusion, our results suggest that OT mRNA synthesis in human amnion/chorion/decidua is regulated by estrogen, and that the estradiol-induced increase in OT mRNA is a receptor-mediated event involving increased transcription. Our results also demonstrate that both E_R and P_R mRNA are expressed in amnion, chorion and decidua and that ER mRNA increases significantly around the time of labor onset. These findings support our hypothesis that the timing of human parturition may be regulated by a paracrine network, within intrauterine tissues, that involves OT, estrogen and progesterone. Further study of this network may yield important clues towards a better understanding of the physiology and pathophysiology of human parturition.

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FIGURE LEGENDS

Figure 1. Ribonuclease protection assays showing the effects of estrogen and progesterone on OT gene expression in human chorio-decidua. All explants were first incubated for 12 hours in medium alone prior to steroid hormone addition. **A.** Explants of chorio-decidua were incubated for 12 additional hours in the absence (C) or presence of increasing concentrations of estradiol-17β (lane 1, 1 nM; 2, 10 nM; 3, 100 nM; 4, 1000 nM) P is the probe lane. The upper panel demonstrates protection of the OT probe. The lower panel illustrates γ-actin to confirm equal amounts of RNA in each lane.There is a significant increase in OT mRNA with 1 and 10 nM estradiol but higher concentrations appear to inhibit OT mRNA synthesis. **B.** Explants of chorio-decidua were incubated in the absence (CO) or presence of estradiol (E1, 1 and E2, 10 nM), progesterone (P1, 1 and P2 100 μM) or a combination of the two higher concentrations of both steroids (EP). The upper panel shows OT mRNA and the lower panel γ-actin. Progesterone appears to have little effect on OT mRNA.

Figure V-1.

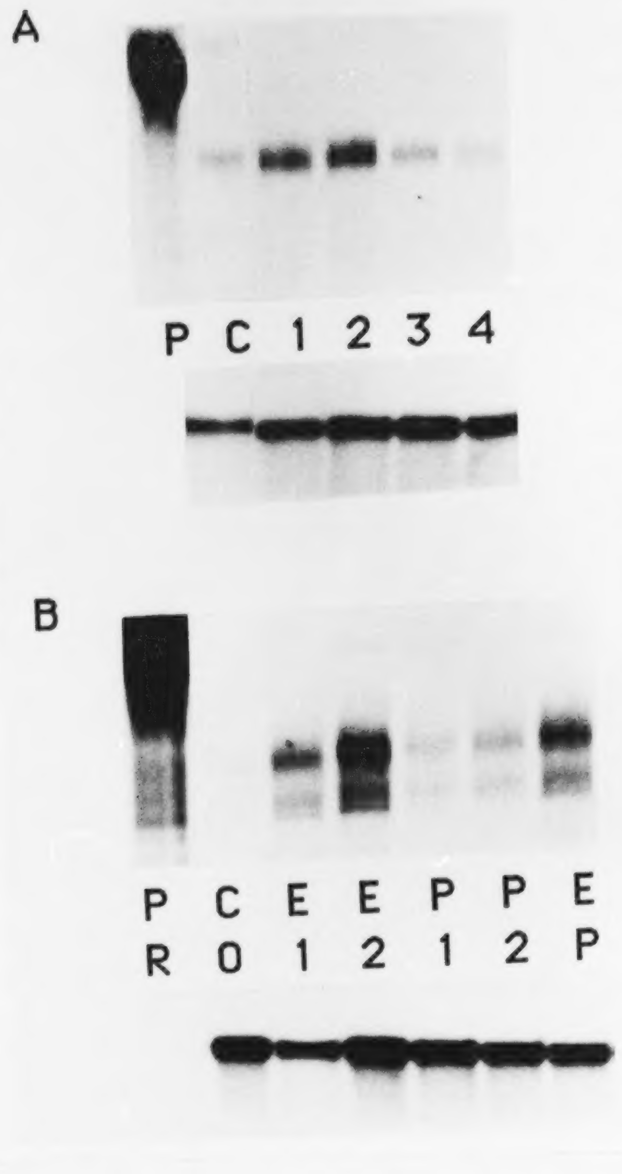


Figure 2. Ribonuclease protection assays demonstrating the effects of tamoxifen on OT mRNA synthesis in chorio-decidua. Explants were incubated for 12 hours alone (CO) or with tamoxifen (T1, 1 and T2, 10 mM), estradiol (E1, 1 and E2, 10 nM) or a combination of the two higher concentrations of both of these agents (TE). PR is the probe lane. The upper panel shows OT mRNA and the lower panel γ-actin. Tamoxifen had little effect alone but inhibited the estradiol-induced increase in OT mRNA.

Figure V-2.

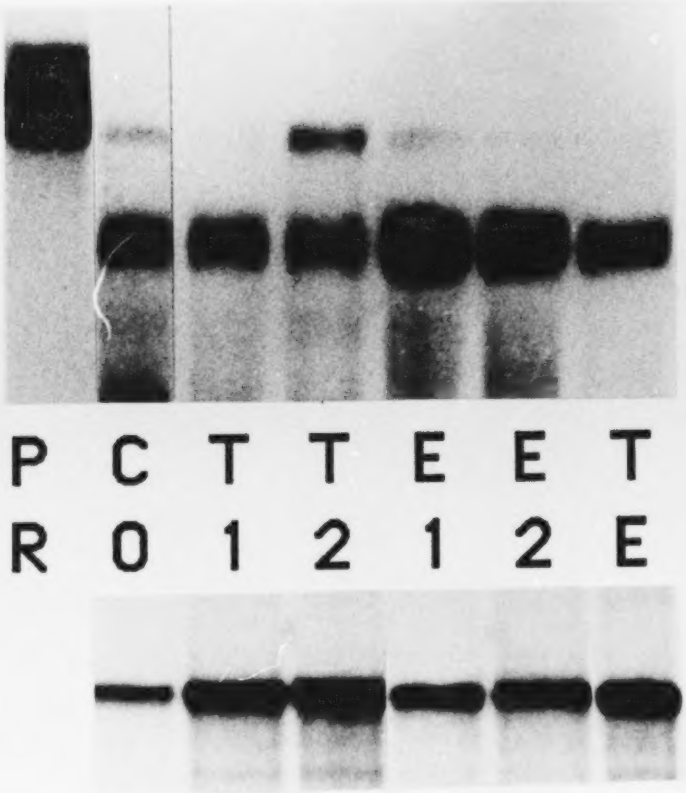


Figure 3. Ribonuclease protection assays demonstrating the effects of cycloheximide and α-amanitin on estradiol-induced OT mRNA synthesis in chorio-decidua. Explants were incubated for 12 hours alone (CO) or with 10 nM estradiol (E), 10 μM cycloheximide (CX), 100nM α-amanitin (AM) or a combination of estradiol with cycloheximide (EC) or α-amanitin (EA). PR is the probe lane. The upper panel shows OT mRNA and the lower panel γ-actin. Both estradiol and cycloheximide stimulated OT mRNA synthesis and their effects were additive. There was little effect of α-amanitin alone but it partially blocked the estradiol-induced increase in OT mRNA.

Figure V-3.

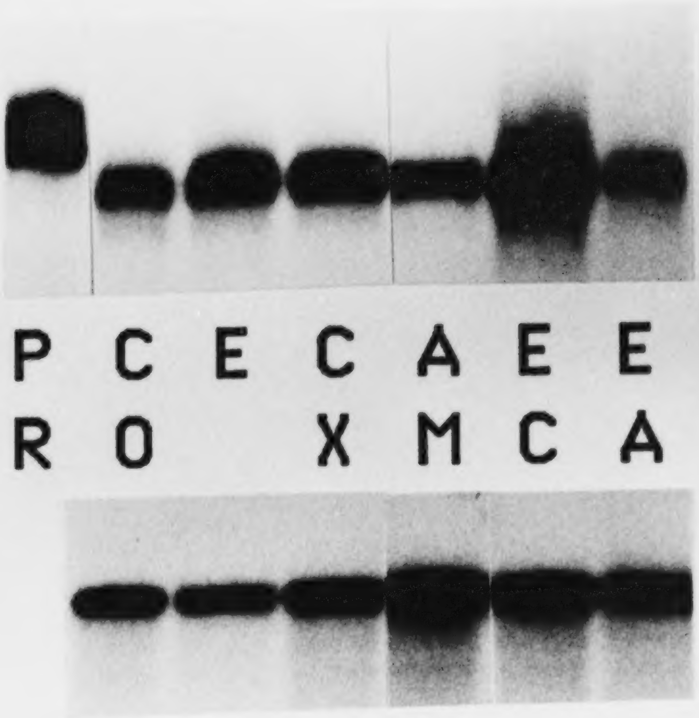


Figure 4. Ribonuclease protection assays demonstrating the presence of mRNA for estradiol and progesterone receptors in human intrauterine tissues. Tissues were obtained immediately following delivery and total RNA extracted. Protection assays were conducted in the presence of cRNA probes for the estradiol receptor (**panel A**) or the progesterone receptor (**panel B**). The tissues assessed include placenta (PL), amnion (AM), chorion (CH), decidua (DE) and, for comparative purposes, tissue from a leiomyoma (LE). **Panel C** shows the γ-actin mRNA content for standardization. All tissues contained mRNA for both receptors with lowest amounts in placenta and highest amounts in decidua. The progesterone receptor probe protected two discrete bands in each tissue.

Figure V-4.

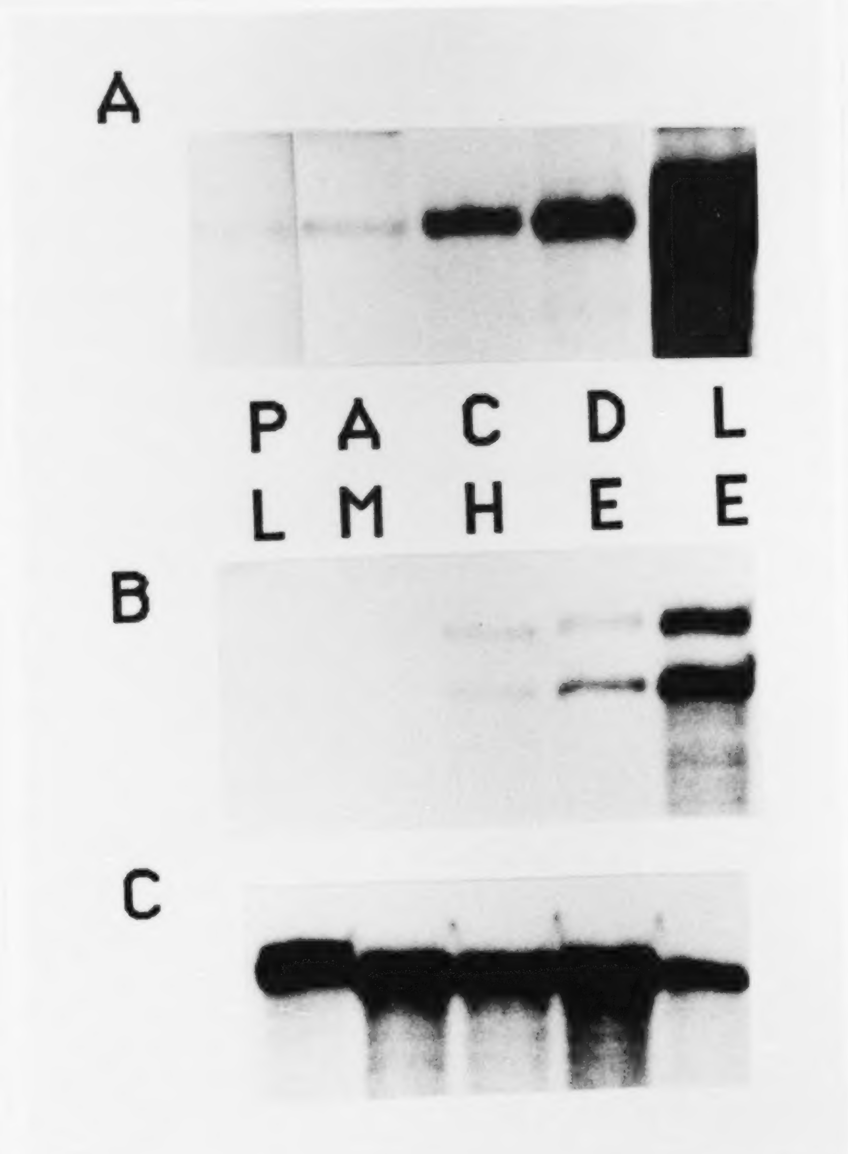
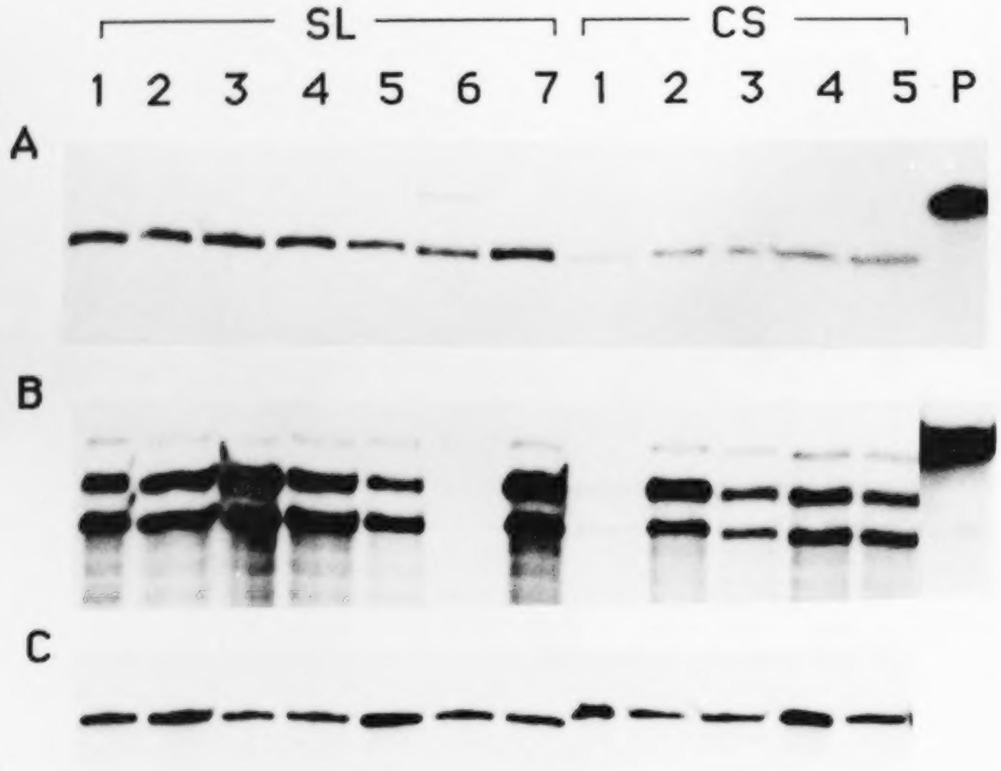


Figure 5. Human chorio-decidual tissues were obtained at term immediately following spontaneous labour and vaginal delivery (SL group, n = 7) or after elective repeat cesarean section (CS group, n = 5). Total RNA was extracted and ribonuclease protection assays were run using cRNA probes for the estrogen receptor (E_R, panel A), the progesterone receptor (P_R, panel B) or γ-actin (panel C). RNA was quantified by spectrophotometry and equal amounts applied for each sample. All tissues were positive for E_R and there was significantly greater E_R mRNA in the tissues obtained following spontaneous labour. Panel C confirms that similar amounts of RNA were applied to each lane. The symbol P denotes the probe.

Figure V-5.



CHAPTER VI

SUMMARY AND CONCLUSIONS

Oxytocin is an endocrine/paracrine hormone involved in many central and peripheral reproductive functions. The synthesis and release of OT appears to be influenced by circulating gonadal steroids. In this thesis I have examined the synthesis and regulation of OT mRNA by gonadal steroids: a) during pubertal maturation in the rat hypothalamus (where OT is considered to have an endocrine function) and b) in human fetal membranes around the time of parturition (where OT may have a paracrine function).

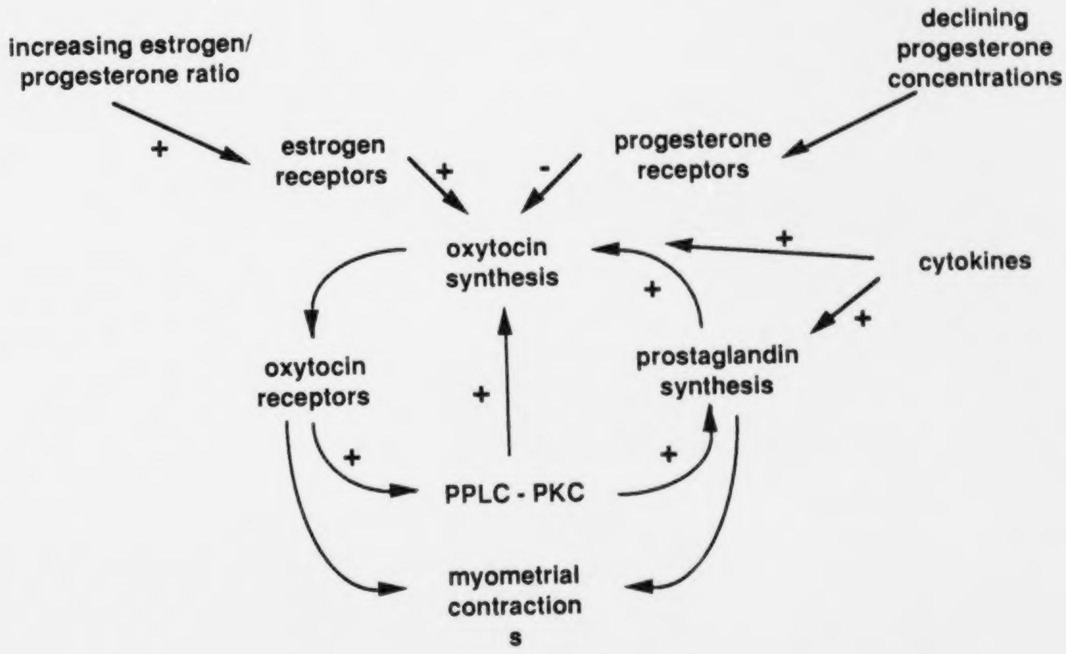
Oxytocin has been demonstrated to increase during pubertal maturation in several species. Factors that control the onset of puberty are incompletely understood but involve a reactivation of the hypothalamo-pituitary-gonadal axis. The increased secretion of gonadal steroids, which coincides with this reactivation, subsequently influences the development of reproductive functions and behaviour. In both male and female rats, the levels of endogenous circulating estradiol and testosterone increases during puberty. Gonadal steroids stimulate the release of OT centrally, as well as into the peripheral circulation, and influence the electrical activity of oxytocinergic neurons. The OT promoter region contains an estrogen responsive element capable of conferring estradiol sensitivity on a heterologous reporter gene in cells expressing the estradiol receptor.

In the initial studies (chapter II), I examined the endocrine role of sex steroids on OT gene expression in male and female rats around the time of pubertal development. The results show that: 1) neural oxytocin mRNA increases 5 to 10-fold in both sexes during puberty; 2) the pubertal upregulation of oxytocin mRNA was largely but not completely inhibited by prepubescent gonadectomy, indicating a requirement for intact gonads as well as some other as-yet-undefined factor(s); 3) pubertal treatment of gonadectomized animals with estradiol or testosterone abolished the effects of gonadectomy suggesting that sex steroids are involved in upregulation of OT mRNA during pubertal maturation; 4) treatment of prepubertal animals with estradiol or testosterone from P10 to P20 had no effect on oxytocin mRNA levels, suggesting that neural maturation or other factors are necessary requisites for steroid sensitivity; 5) mature male rats and females at all stages of the estrous cycle expressed similar levels of neural oxytocin mRNA, suggesting that cyclic variation in estradiol during the estrous cycle in female rats does not affect OT gene expression and that the lowest normal physiological level of estrogen (eg. during diestrous) is sufficient to maintain mature amounts of oxytocin mRNA; and 6) activin mRNA was expressed at similar levels in all brain regions, and did not vary as a function of gonadectomy, or steroid treatment, making it unlikely that activin mediates the observed changes in OT mRNA synthesis. Together, these data indicate that neural oxytocin mRNA is induced by gonadal steroids during puberty, and possibly provide a mechanism for coordinating the development of reproductive functions with other pubertal changes.

In 1906 the first function of OT discovered was contractile effect on uterine myometrium. Oxytocin is well documented to be a potent and effective myometrial stimulant that is widely used to induce and augment labour. However, an endocrine role for OT in the initiation of human parturition remains controversial. The balance of evidence suggests that neural OT plays only a facilitatory role during the second stage of labour and after delivery but not in the actual initiation process itself. We and others have proposed that human labour may be regulated by factors synthesized within the fetal membranes (amnion, chorion) and maternal decidua. Amnion, chorion and decidua have substantial capacity to synthesize various peptides, steroids and prostanoids, adjacent to the likely target organ of these compounds, the myometrium.

Therefore, in the second set of studies (chapters IV and V) we explored a novel hypothesis addressing a potential paracrine role for oxytocin in the regulation of human parturition. These studies were initiated to explore the possibility of local synthesis of oxytocin in human fetal membranes (amnion and chorion) and maternal decidua. The experiments revealed several lines of evidence consistent with the hypothesis (schematic Figure VI-1) that OT is synthesized in intrauterine tissues and may play a role in the initiation of human labour in a paracrine manner. First, by Northern blot analysis, ribonuclease protection assay and *in situ* hybridization, we found that the OT gene is expressed in amnion, chorion and decidua. Levels were highest in decidua, the region

Figure VI-1. Hypothesized decidua-myometrial paracrine system



immediately adjacent to the putative target organ, the myometrium. Although the transcript size was 60-80 nucleotides smaller in decidua than the transcripts in amnion and chorion, no evidence of splice variation was detected. The OT precursor mRNA detected in these tissues includes the region that encodes for the OT peptide and the difference in transcript size may be due to variation in the poly A tail length. Second, a 3-4 fold increase in OT gene expression occurring in chorio-decidua following labour and delivery strengthens our hypothesis that local synthesis of OT could stimulate uterine contractions in a paracrine fashion without increasing circulating maternal OT concentrations. Third, estradiol increased OT mRNA levels in *in vitro* full thickness explants (amnion, chorion, decidua) obtained from prelabour tissues while progesterone partially prevented the estradiol induced increase in OT mRNA. Fourth, The effects of estradiol on OT mRNA in intrauterine tissues are receptor mediated and appear to be exerted at the transcriptional level. Fifth, estradiol and progesterone receptor mRNA are expressed in amnion, chorion and decidua with the highest expression in decidua. Finally, there is a significant increase in estradiol receptor gene expression around the time of parturition. Together these results suggest an important paracrine/autocrine role for OT and estradiol in the physiology of human parturition. These results strongly suggest the existence of an intrauterine paracrine system with positive/negative interactions among OT, estradiol and progesterone that is involved in the maintenance of pregnancy and initiation of parturition. A change in the local estrogen/progesterone ratio (in favour of estradiol) or an increase in estradiol receptors may stimulate OT synthesis and the onset of labour. Premature labour is a major contributing factor to perinatal mortality and morbidity. This information may be a step towards understanding the physiology of human parturition and lead to new strategies in developing better methods for preventing or treating premature labour.